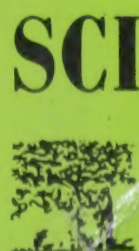


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## Heat Denaturation of Rabbit Skeletal G-Actin in the Presence of ATP

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### ABSTRACT

*Heat treatments at pH 7.5 induced the polymerisation of G-actin without the addition of KCl or MgCl<sub>2</sub>. This heat-induced polymerisation of G-actin was accompanied by the splitting of ATP. The polymerised actin at 45°C could be depolymerised by dialysis against a solution containing ATP. Electron microscopic observation of actin showed that irregular filaments were formed after heat treatment at 45°C for 3 h, and at 55°C small filamentous pieces were formed. The abilities of heavy meromyosin ATPase (EC 3.6.1.32) activation and ATP splitting of G-actin were drastically impaired by heating to temperatures above 50°C. Studies of the temperature dependence of fluorescence and viscosity data also revealed that heat denaturation of G-actin in the presence of ATP occurred steeply between 45°C and 55°C.*

**Key words:** G-actin, heat denaturation, polymerisation, actin filament, ATP.

### INTRODUCTION

Heat treatments routinely used for the processing of meat products such as sausages could produce substantial protein denaturation, which is critical to heat-induced gelation of muscle proteins (Yasui and Ishioroshi 1980). Knowledge of heat-induced

denaturation of the major muscle proteins, myosin and actin, is therefore necessary for an understanding of the mechanism of heat gelation of the cooked meat products. Heat denaturation of myosin, which is considered to be the most effective protein for gel formation, has been studied by many workers (Takahashi *et al* 1962; Kawakami *et al* 1971). The present authors' research has been devoted to the study of heat denaturation of another major muscle protein, actin (Ikeuchi *et al* 1981).

Actin undergoes transformation from a monomeric species (G-actin) to a long, helical polymer (F-actin). This conversion of G- to F-actin induced by the addition of neutral salts is coupled with dephosphorylation of ATP into ADP and inorganic phosphate. The G-F transformation can be reversibly repeated by cycling the proper salt concentration in the presence of ATP (Laki *et al* 1950; Straub and Feuer 1950). Since it is well known that G-actin is not stable in the absence of nucleotides and divalent cations, resulting in irreversible denaturation (Asakura 1961; Martonosi and Gouvea 1961), it has been considered that the binding of ATP to G-actin contributes to the promotion of polymerisation and the stabilisation of actin structure (Kasai *et al* 1965; Cooke 1975). Concerning the effect of ATP on the denaturation of actin, Lehrer and Kerwar (1972) reported from experiments on the temperature dependence of fluorescence that a protective effect of ATP on the structure and function of G-actin diminished above 40°C, while F-actin denatured upon heat treatments above 50°C. In a previous paper (Ikeuchi *et al* 1981), the authors also demonstrated that the heat denaturation of F-actin obeyed first-order kinetics, and ATP evidently acted as a protector against heat denaturation. These results suggest that the molecular response of G-actin to heating is different from that of F-actin.

The objective of this work was to examine the heat-induced denaturation of G-actin in the presence of ATP. Estimates of the heat denaturation of G-actin were made by measurements of polymerisation, ATP splitting and heavy meromyosin (HMM) ATPase (EC 3.6.1.32) activating abilities. Structural change of G-actin after heat treatments was also examined by electron microscopy and fluorescence spectrophotometry. Heat conditions were in the temperature range 25–65°C.

## EXPERIMENTAL

### Preparation of G-actin and heavy meromyosin (HMM)

Rabbits were anaesthetised with sodium pentobarbital prior to exsanguination. After cooling the rabbit carcasses in ice-water for a few minutes, *longissimus thoracis*, *semimembranosus* and *biceps femoris* muscles were dissected out from the carcasses for use in the present study. G-actin was prepared according to the procedure of Spudich and Watt (1971). HMM was obtained by the method of Lowey and Cohen (1962) except that ammonium sulphate fractionation was made between 42 and 58% w/v saturation.

### Heat treatment of G-actin

Heat treatment of G-actin was carried out in 1.5 mg ml<sup>-1</sup> G-actin, 0.1 mM CaCl<sub>2</sub>, 0.5 mM  $\beta$ -mercaptoethanol, either 0.3 mM or 0.9 mM ATP and 10 mM Tris-HCl (pH

7.5) at varying temperatures. The pH of Tris-HCl buffer decreased about 0.3 unit with increasing temperature from 25°C to 65°C, so that a correction of pH value was made for each buffer.

It was confirmed by sodium dodecyl sulphate gel electrophoresis that there was no sign of proteolysis of G-actin during heat treatments.

### Estimation of actin denaturation

#### *Viscosity measurement*

Viscosity measurements were carried out with an Ostwald-type viscometer of 10 ml capacity having a water flow time of about 120 s at 25°C. The results were expressed as the specific viscosity ( $\eta_{sp}$ ).

#### *ATP splitting ability*

In order to determine the splitting of ATP by G-actin during heat treatment, G-actin solutions were incubated for the designated times at varying temperatures. After the incubation, an equal volume of cold 10% w/v trichloroacetic acid was added to the solutions and the precipitated actin was immediately removed by centrifugation. It should be noted that dephosphorylation of ATP by heating did not occur under the conditions employed.

#### *HMM ATPase (EC 3.6.1.32) activating ability*

The remaining HMM ATPase activating ability of actin was measured at 30°C by incubating the heat-treated G-actin with intact HMM. Prior to measuring the ATPase, the heat-treated G-actin was polymerised to F-actin by adding KCl (final concentration 50 mM) and then leaving to stand for 6 h at 0°C. The remaining activity was expressed as relative activity (%) versus the activity activated by native F-actin polymerised with salt (50 mM KCl).

#### *Fluorescence analysis*

Fluorescence emission spectra were obtained with a Hitachi F-3010 spectrofluorometer. The G-actin samples ( $0.2 \text{ mg ml}^{-1}$ ) after heat treatment were excited at 280 nm, and emission was recorded at right angles to the excitation over the range 280–420 nm. The solution temperature was controlled with a thermostated sample housing.

#### *Electron microscopic observation*

For electron microscopic study, G-actin solutions were diluted to  $0.05 \text{ mg ml}^{-1}$ . Actin samples were negatively stained with 1.0% w/v uranyl acetate according to the method of Huxley (1963). The samples were observed under a Hitachi H-300S electron microscope operated at 75 kV.

### Inorganic phosphate determination

The inorganic phosphate liberated after the reactions of actin-activated HMM ATPase and of ATP-splitting by G-actin was determined by the method of Fiske and Subbarow (1925).

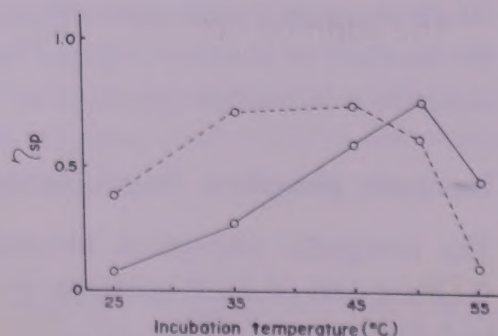
### Protein concentration

Protein concentration was determined by the biuret reaction (Gornall *et al* 1949) which had been standardised with bovine serum albumin.

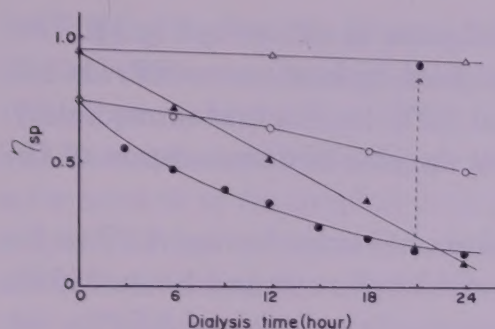
## RESULTS AND DISCUSSION

Figure 1 shows the specific viscosity of G-actin after heat treatment at varying temperatures. As can be seen in this figure, specific viscosity of G-actin increased with increasing temperature in the presence of 0.3 mM ATP: the viscosity of G-actin attained its maximum value ( $\eta_{sp} = 0.75$ ) after incubation at 35–45°C for 3 h or at 50°C for 30 min. This value was about 25 % smaller than that of F-actin ( $\eta_{sp} = 0.98$ ) obtained by adding 50 mM KCl to native G-actin. Swezey and Somero (1982) suggested that the conservation of an ability to polymerise was achieved by alterations in the types of bond and changes in the energy costs of altering actin conformation during the assembly process. The present result therefore suggests that the conversion of G-actin to filamentous actin induced by heat treatment is due to the change in energetics of G-actin as described above, and the conversion is incomplete or irregular in shape when compared with the usual polymerisation reaction induced by the addition of neutral salt (Figs 1, 2 and 7). In addition, the rate of viscosity change of G-actin became faster with increasing temperature. Since the G–F equilibrium depends on the temperature, the polymerisation rate naturally increases and the critical concentration of G-actin for polymerisation diminishes on raising the temperature (Oosawa and Kasai 1971). However, an increase in temperature brings about acceleration of the denaturation process, so that the G–F equilibrium would not be set up under the condition of high temperature employed in this experiment.

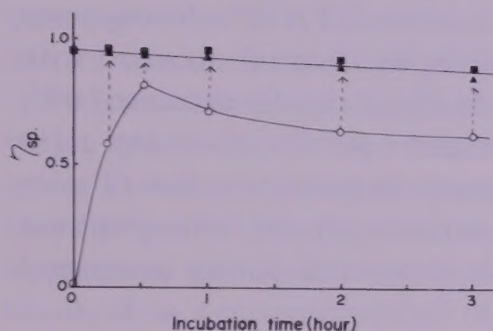
Figure 2 shows the time course of viscosity changes of thermally polymerised actin and native F-actin polymerised with salt during dialysis against a solution containing 2 mM Tris-HCl (pH 7.5), 0.2 mM ATP, 0.1 mM  $\text{CaCl}_2$  and 0.5 mM  $\beta$ -mercaptoethanol at 0°C. The viscosity of KCl-polymerised native F-actin decreased linearly and rapidly with increase in the dialysing time, whereas the viscosity of thermally polymerised actin decreased slowly. This would appear to be attributable to a difference in the filamentous structure or the length distribution of the polymers between actins polymerised by heating or neutral salt (Fig 7). Upon addition of KCl to the depolymerised G-actin, the viscosity promptly rose again to the level of native



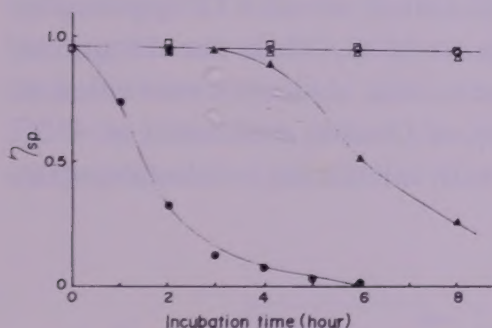
**Fig 1.** Temperature-dependent change of specific viscosity of G-actin. G-Actin ( $1.5 \text{ mg ml}^{-1}$ ) was incubated at various temperatures in the buffer described in 'Experimental'. Solid line, G-actin incubated for 30 min; dotted line, G-actin incubated for 3 h.



**Fig 2.** Reversible G-F transformation of the heat polymerised actin at 45°C for 3 h (○) and native F-actin (△). G-Actin ( $1.5 \text{ mg ml}^{-1}$ ) was polymerised by heating at 45°C for 3 h in the buffer described in 'Experimental'. Native F-actin was made by adding KCl to 50 mM to G-actin. Actins dialysed against a solution containing 0.2 mM ATP, 0.1 mM  $\text{CaCl}_2$ , 0.5 mM  $\beta$ -mercaptoethanol and 2 mM Tris-HCl, pH 8.0 at 0°C (●, ▲); actins incubated at 0°C without dialysis (○, △). Dotted line indicates viscosity change of dialysed actin after the addition of KCl (final concentration 50 mM).



**Fig 3A.** Time course of viscosity changes of the heat-treated G-actin at 50°C for designated times before and after the addition of KCl. ○, Viscosity right after heat treatment at 50°C; ●, viscosity after the addition of KCl (final concentration 50 mM) followed by 6 h incubation at 0°C (■) or 20°C (▲). Dotted lines indicate the viscosity change induced by the addition of KCl.



**Fig 3B.** Time course of viscosity changes of the heat-treated G-actin at varying temperatures for designated times after the addition of KCl (final concentration 50 mM). ○, 25°C; □, 35°C; △, 45°C; ▲, 50°C; ● 55°C.

F-actin (see dotted line in Fig 2). Oosawa and Maruyama (1986) found that the change in the polymerisation rate of G-actin incubated at 25°C for 24 h was reversible by storing the sample at 0°C. This phenomenon may be the case for the G-actin incubated at 45°C for 3 h. Moreover, it was observed that the viscosity of thermally polymerised actin decreased slowly even without dialysis against ATP solution at 0°C (see open circle symbols in Fig 2). Asakura *et al* (1960) reported a similar temperature-dependent depolymerisation of  $\text{Mg}^{2+}$  polymer: F-actin polymer formed by the addition of  $\text{MgCl}_2$  was depolymerised by decreasing temperature.

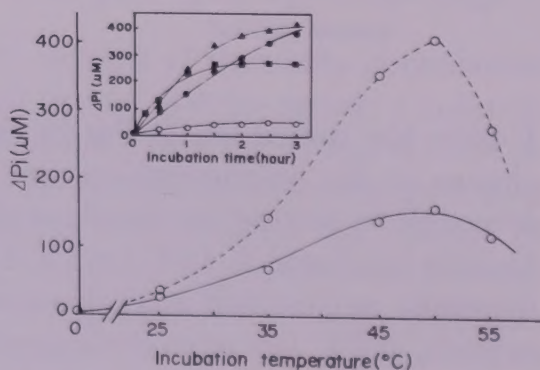
The addition of KCl to thermally polymerised actin (at 50°C) brought about further increase in the viscosity (Fig 3A), suggesting that the polymerisation of G-actin induced by heating is incomplete and the actin polymers still have salt-dependent polymerising ability as native actin does (Oosawa and Maruyama 1986). Of course, the result shown in Fig 3A opens the possibility that the actin is essentially completely polymerised by heating (that is, the monomer concentration is low) and a rise in viscosity on adding salt is caused by a change in the length distribution of the polymers. The time course of viscosity change of the thermally

treated G-actin after the addition of KCl was investigated as shown in Fig 3B. The salt-dependent polymerising ability was maintained even by heat treatments for 8 h below 45°C, but it was rapidly lost after heating at 50°C for 3 h and immediately after heating at 55°C. This result demonstrates that the rate of denaturation of G-actin rises steeply between 45°C and 55°C.

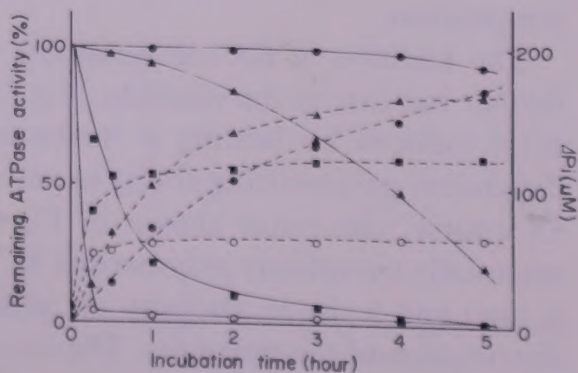
It is well established that reversible polymerisation of G-actin having ATP to F-actin having ADP is accompanied by the splitting of ATP (Straub and Feuer 1950). Therefore, the effect of various heating temperatures on the splitting of ATP by G-actin was investigated in the presence of 0.3 mM or 0.9 mM ATP. As shown in Fig 4, the extent of ATP splitting by G-actin increased with increasing temperature up to 50°C and then decreased. The investigation of the time course of ATP splitting (inset of Fig 4) revealed that the initial rate of ATP splitting by G-actin increased with increasing temperature up to 55°C and the rates gradually or rapidly decreased with increasing time of incubation. At the higher temperature (65°C), it was lost right after the initiation of incubation. These results clearly demonstrate that G-actin molecules split ATP into ADP and Pi during heat-induced polymerisation without the addition of neutral salt, unless the G-actin is denatured during prolonged heating at such a temperature.

Figure 5 shows the relation of HMM ATPase activating ability and ATP splitting ability of G-actin during heat treatments above 45°C. Initial rate of ATP splitting by G-actin increased with increasing temperature up to 55°C, while the remaining HMM ATPase activity rapidly decreased except in the case of G-actin incubated at 45°C. In particular, the ATPase-activating activity of G-actin incubated at 45°C was the same as that of native F-actin polymerised with salt during incubation up to

**Fig 4.** Time-dependent ATP splitting of G-actin. G-actins were treated at varying temperatures for 3 h. Solid line, G-actin in the presence of 0.3 mM ATP; dotted line, G-actin in the presence of 0.9 mM ATP. Inset indicates time course of ATP splitting by G-actin in the presence of 0.9 mM ATP. ●, 45°C; ▲, 50°C; ■, 55°C; ○, 65°C.

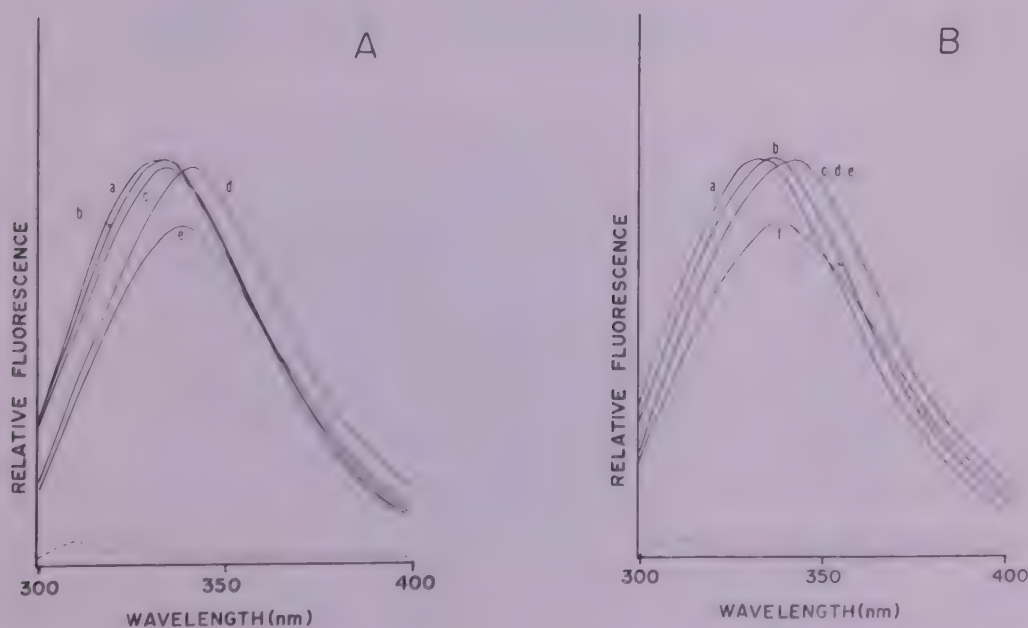


**Fig 5.** Time courses of ATP splitting (dotted line) and remaining HMM ATPase activity (solid line) by G-actin treated at varying temperatures. ●, 45°C; ▲, 50°C; ■, 55°C; ○, 65°C.



3 h, suggesting that the G-actin fully retains its biochemical property. At 65°C both HMM ATPase activating ability and ATP splitting ability were almost lost within 15 min, probably because of the instantaneous denaturation of G-actin (Figs 5 and 6B). As can be seen in this figure, decrease in HMM ATPase activating ability by G-actin seemed to be coupled with a decrease in the rate of ATP splitting. Since the polymerisation rate increases with increasing temperature and reaches a maximum at a critical temperature where denaturation reaction becomes predominant (Fig 1), the polymerisation reaction probably proceeds until G-actin loses its ATPase activity. Furthermore, it is worth noting that these changes happened even in the presence of a sufficient amount of ATP. Accordingly, it may be concluded that a protective effect of ATP on heat denaturation of G-actin is not obvious unlike the case of F-actin (Lehrer and Kerwar 1972; Ikeuchi *et al* 1981).

The conformational change of G-actin induced by heat treatment was examined with a spectrofluorometer. Figure 6 shows the fluorescence emission spectra of G-actins heated at varying temperatures for 3 h (Fig 6A) and at 65°C for designated times (Fig 6B). The fluorescence obtained by exciting at 280 nm is mainly from tryptophan with lesser contributions from tyrosine (Weber and Young 1964). The results show a progressive shift of the peak to longer wavelength (so called red shift) with increasing temperature (Fig 6A) and incubation time at 65°C (Fig 6B), indicating that G-actin molecules undergo some conformational changes during heating. However, differences in the fluorescence peak wavelength and fluorescence intensity between 0 and 45°C were not as large as expected (0°C, 331.8 nm; 45°C, 332.0 nm). Similarly, when the change in fluorescence of G-actin incubated at 45°C was compared with that of native F-actin, there was a small difference in the

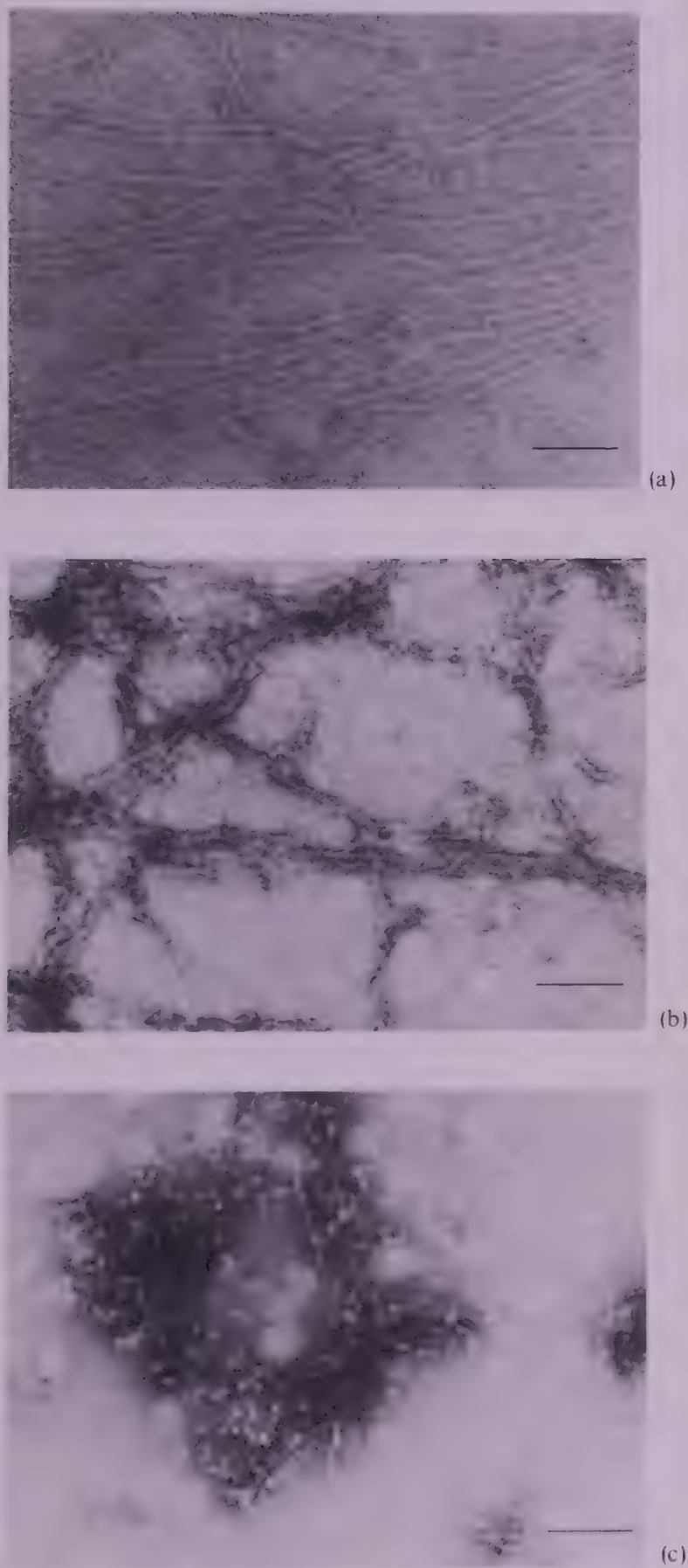


**Fig 6.** (A) Fluorescence emission spectra of G-actin treated at varying temperatures for 3 h. Excitation wavelength was 280 nm. The dotted line is buffer blank. (a) Native F-actin, (b) G-actin at 0°C, (c) G-actin at 45°C, (d) G-actin at 55°C, (e) G-actin at 65°C. (B) Fluorescence emission spectra of G-actin treated at 65°C for designated times. (a) No heating (control), (b) 1 min, (c) 5 min, (d) 30 min, (e) 60 min, (f) 3 h. The dotted line is buffer blank.

fluorescence peak wavelength between their actins (native F-actin 330.7 nm). This slight conformational change at 45 °C may be reflecting intermediate stages leading to the denatured state (Oosawa and Maruyama 1986). Heating at 65 °C for 3 h led to a significant decrease in the intensity, which may be attributed to quenching of fluorescence due to the slight development of turbidity in the sample. In addition it was observed that an irreversible conformational change of G-actin instantaneously occurred when heated at 65 °C (Fig 6B). This was consistent with the result that the ATP splitting ability of G-actin heated at 65 °C was lost within 15 min (Fig 5). The results shown in Figs 5 and 6 also support the view that heat denaturation of G-actin occurs steeply between 45 °C and 55 °C (Fig 3B, Ikeuchi *et al* 1981).

Finally, the ultrastructural change of G-actin induced by heat treatment was examined with an electron microscope (Fig 7). G-actin heated at 45 °C for 3 h formed filaments which were somewhat collapsed into shorter fragments and aggregated in a disordered way (Fig 7b) when compared with F-actin polymerised by the addition of KCl (Fig 7a). Heat treatment at 55 °C for 3 h converted its irregular filaments into small curved filamentous pieces or lumps of them (Fig 7c). It can be presumed from these results that, in the case of heat treatment at moderate temperature (45 °C), where the polymerisation reaction proceeds faster than denaturation reaction does, G-actin molecules do not always retain the specificity for binding to adjacent G-actin, so that the resulting polymer becomes an irregular structure (Fig 4b). At a higher temperature (55 °C), where the denaturation reaction occurs much faster than the polymerisation reaction, the G-actin molecules bind randomly to each other through non-specific site(s), forming small filamentous pieces (Fig 7c). In the case of the polymerisation reaction induced by the addition of neutral salt at low temperature (0 °C), on the other hand, the constituent G-actin molecules bind each other through specific binding site(s), resulting in the formation of F-actin having a regular structure, ie a straight filament with double strands (Fig 7a, Hanson and Lowy 1963). It was regrettable that the present authors could not clarify a difference in the fine structure between actins polymerised by heating or salt from the present microscopic observation (eg whether or not the heat induced polymers are helical). Speculations on the mechanism of heat polymerisation described above have therefore to be postponed until some structural properties are established. One obvious future avenue will be indicated by a comparison of the optical diffraction patterns.

In summarising the results of the present study, G-actin at pH 7.5 was transformed into an irregular filamentous structure during heat treatment, which was accompanied by ATP splitting, even if neutral salts were absent. This thermally polymerised actin was depolymerised by dialysis against a solution containing ATP. When KCl was added to the depolymerised actin, it was fully polymerisable again. However, this reversible G  $\rightarrow$  F transformation could no longer take place if G-actin began to lose ATP splitting ability while it was exposed to high temperature. It has also become apparent that the rate of heat denaturation of G-actin in the presence of ATP would rise steeply between 45 °C and 55 °C. Furthermore, G-actin incubated at 45 °C for 3 h was somewhat different from native F-actin polymerised with salt in physicochemical and structural properties although it retained its biochemical property fully.



**Fig 7.** Electron micrographs of native F-actin and heat-treated G-actins. (a) F-actin polymerised by the addition of KCl. (b) G-actin treated at 45 C for 3 h. (c) G-actin treated at 55 C for 3 h. The scale bar represents 0.1  $\mu$ m.

## ACKNOWLEDGEMENT

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## REFERENCES

- Asakura A 1961 The interaction between G-actin and ATP. *Arch Biochem Biophys* **92** 140–149.
- Asakura A, Kasai M, Oosawa F 1960 The effect of temperature on the equilibrium state of actin solutions. *J Polymer Sci* **44** 35–49.
- Cooke R 1975 The role of the bound nucleotide in the polymerization of actin. *Biochemistry* **14** 3250–3256.
- Fiske C H, Subbarow Y 1925 The colorimetric determination of phosphorus. *J Biol Chem* **66** 375–400.
- Gornall A G, Bardawill C T, David M M 1949 Determination of serum protein by means of the biuret reaction. *J Biol Chem* **177** 751–766.
- Hanson J, Lowy J 1963 The structure of F-actin and of actin filaments isolated from muscle. *J Mol Biol* **6** 46–60.
- Huxley H E 1963 Electron microscope studies on the structure of natural and synthetic protein filaments from striated muscle. *J Mol Biol* **7** 281–308.
- Ikeuchi Y, Ito T, Fukazawa T 1981 A kinetic analysis of thermal denaturation of F-actin. *Int J Biochem* **13** 1065–1069.
- Kasai M, Nakano E, Oosawa F 1965 Polymerization of actin free from nucleotides and divalent cations. *Biochim Biophys Acta* **94** 494–503.
- Kawakami H, Morita J, Takahashi K, Yasui T 1971 Thermal denaturation of myosin, heavy meromyosin and subfragment 1. *J Biochem* **70** 635–648.
- Laki K, Bowen W J, Clark A M 1950 The polymerization of proteins. Adenosinetriphosphate and the polymerization of actin. *J Gen Physiol* **33** 437–443.
- Lehrer S S, Kerwar G 1972 Intrinsic fluorescence of actin. *Biochemistry* **11** 1211–1217.
- Lowey S, Cohen C 1962 Studies on the structure of myosin. *J Mol Biol* **4** 293–308.
- Martonosi A, Gouvea M A 1961 Studies on actin. VI. The interaction of nucleotide triphosphates with actin. *J Biol Chem* **236** 1345–1351.
- Oosawa F, Kasai M 1971 Actin. In: *Subunits in Biological Systems*, eds Timasheff S N & Fasman G D. Marcel Dekker, New York, pp 261–322.
- Oosawa M, Maruyama K 1986 An intermediate state of G-actin between native and denatured: polymerization rate decreases but extent of polymerization remains unchanged. *J Biochem* **100** 1001–1008.
- Spudich J A, Watt S 1971 The regulation of rabbit skeletal muscle contraction. *J Biol Chem* **246** 4866–4871.
- Straub F, Feuer G 1950 Adenosine triphosphate, the functional group of actin. *Biochim Biophys Acta* **4** 455–470.
- Swezey R R, Somero G N 1982 Polymerization thermodynamics and structural stabilities of skeletal muscle actins from vertebrates adapted to different temperatures and hydrostatic pressures. *Biochemistry* **21** 4496–4503.
- Takahashi K, Yasui T, Hashimoto Y, Tonomura Y 1962 Physico-chemical studies on denaturation of myosin adenosinetriphosphatase. II. Changes in chromatographic profile and optical rotation. *Arch Biochem Biophys* **99** 45–51.
- Weber G, Young L B 1964 Fragmentation of bovine serum albumin by pepsin. *J Biol Chem* **239** 1424–1431.
- Yasui T, Ishioroshi M 1980 Heat-induced gelation of myosin in the presence of actin. *J Food Biochem* **4** 61–78.

# Wheat-bug Damage in New Zealand Wheats: the Feeding Mechanism of *Nysius huttoni* and its Effect on the Morphological and Physiological Development of Wheat

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## ABSTRACT

Adult *Nysius huttoni* White were placed in separate cages containing cultivars Rongotea and Karamu at the late anthesis, watery ripe and milky ripe stages of development. Between 84 and 99% of the matured kernels were injured with the characteristic visible markings of bug-damaged wheat. All the samples contained strong wheat-bug proteinase activity as shown by the incubated SDS-sedimentation test and the disappearance of HMW glutenin subunits from electrophoretograms. Grain infested at late anthesis was most severely affected with shrivelled kernels, high screenings, protein, free amino acids and  $\alpha$ -amylase, and low kernel weight, germination capacity and carbohydrate content. Grain infested at the watery ripe and milky ripe stages had values for the above properties closer to uninfested control wheat values.

It was suggested that *N. huttoni* sucked sap from lateral sieve tubes in the wheat ovary at late anthesis causing severe disruption to physiological development of the grain. Two commercial lines of wheat showed some characteristics of this effect.

**Key words:** Bug damage, *Nysius huttoni*, wheat-bug, growth stage, wheat development, protein, carbohydrate,  $\alpha$ -amylase, proteinase, electrophoresis, SDS-sedimentation test, kernel weight, screenings, germination, wheat-bug feeding, insect cages.

## INTRODUCTION

In parts of Europe, Asia and North Africa the wheat bugs *Eurygaster* and *Aelia* secrete salivary juices into developing wheat kernels and feed on the solubilised contents of the grain. Kent-Jones and Amos (1938) suggested that if the wheat-bug attacked very immature grain its development would be more severely affected than wheat attacked at a fairly mature stage. Tischler (1939) showed that early attack produced shrivelled grain and late attack had little effect on grain size and weight, the only visible effect being the appearance of dark spots with a surrounding pale area. The properties of these bug-damaged wheats are altered in several other respects: reduced total protein content, increased water, trichloroacetic acid (TCA) and alcohol soluble nitrogen, increased protease and amylase activity (Kretovich 1944), and reduced germination capacity (Merenet 1936; Tischler 1939).

In New Zealand the insect most suspected of causing bug damage to wheat is *Nysius huttoni* White (Morrison 1938; Cressey *et al* 1987; Every *et al* 1989). When wheat at the watery ripe stage of development was experimentally infested by *Nysius*, the mature grain was shown to have increased protease activity (Cressey *et al* 1987), unchanged  $\alpha$ -amylase activity, slightly reduced kernel weight and unchanged protein and ash content (Lorenz and Meredith 1988). Three commercial lines of bug-damaged wheat in New Zealand have been studied where the insect species causing the damage and the development stage of the wheat when the insect attacked the grain were unknown. All samples had increased protease activity, two samples had substantially elevated TCA-soluble nitrogen (Cressey and McStay 1987a) and one sample had elevated  $\alpha$ -amylase activity (Lorenz and Meredith 1988). The level of  $\alpha$ -amylase in the other two samples has not been reported. Cressey and McStay (1987a) suggested that the high levels of amino acids (TCA soluble nitrogen) were caused by the insect attack halting or slowing the physiological development of the grain. In contrast, the high amino acid levels in grain attacked by *Eurygaster* in Russia were explained by the action of insect protease on wheat gluten protein (Kretovich 1944).

The aim of this study is to determine whether the physico-chemical changes of bug-damaged wheat discussed above result from interrupted physiological development of grain or the action of insect enzymes on grain constituents. These properties were analysed in two New Zealand wheat cultivars which were infested by caged *N. huttoni* during three stages of wheat development: late flowering (anthesis), watery ripe and milky ripe. The mechanism of wheat-bug feeding is discussed in relation to the physico-chemical changes in wheat infested at the most immature stage.

## EXPERIMENTAL

### Wheat and flour samples

Three commercially grown bug-damaged samples detected by the Wheat Research Institute harvest evaluation testbake were examined: one line of cultivar Oroua grown in Mid-Canterbury in the 1983/84 season, one line of cultivar Kopara

grown in Central Otago in the 1985/86 season, and one line of cultivar Aotea (season unknown). One good bread-making cultivar, Rongotea, and one poor bread-making cultivar, Karamu, were used in the caging experiment.

The bug-damaged Aotea sample was only available as a flour. All other samples were prepared as wholemeal flour on a falling number mill type 3100 for the various analytical tests.

### **Caging insects on wheat**

Areas of wheat were sown on 1 December 1986 at the Lincoln DSIR Research Farm. In late January to February 1987 groups of three ears were enclosed in nylon gauze bags and colonised with 50 field-collected *N huttoni* at one of three wheat growth stages: late flowering (GS 69), watery ripe (GS 71) or late milky ripe (GS 77) (Zadoks *et al* 1974). Seven replicates of each growth stage treatment, and three caged groups without *Nysius*, were set up in each cultivar. One month after colonisation a high proportion of adult *Nysius* survived and many nymphs (approximately 30% of the population) had emerged from eggs deposited since colonisation. At this time all cages including controls were sprayed with Pirimicarb ( $12.5 \text{ mg m}^{-2}$ ) to kill insects. Widespread trials have shown that this insecticide causes no impairment of development and quality of wheat. Cages were removed, and the wheat was left to mature before harvest.

### **Screening, kernel weight and visual assessment of grain**

Grain from bagged heads was passed over a screen of 2 mm. Fractions passing (screenings) and retained by (dressed grain) the screens were weighed. All subsequent experiments were performed on dressed grain. Kernel weights and the percentage of damaged kernels were determined for the total dressed sample. The number and location of distinctive insect feeding marks on each kernel were determined for 100 kernels per sample. Damage consisted of an opaque yellow patch usually with a dark puncture mark at the centre.

### **Protein, non-protein nitrogen and carbohydrate**

Total nitrogen was determined by the Kjeldahl procedure by the method of the Association of Official Analytical Chemists (1981). Water-soluble protein was extracted from 1 g wholemeal in 5 ml distilled water and determined by the Coomassie blue G250 (BioRad, Richmond, CA, USA) protein stain method of Bradford (1976). Non-protein nitrogen (6% trichloroacetic acid soluble nitrogen) was determined by the ninhydrin method of Yemm and Cocking (1955) as adapted by Hanford (1967). Total protein was calculated by subtraction of non-protein nitrogen from total nitrogen and multiplication of this value by 5.7.

Total carbohydrate (mainly starch, pentosans and soluble sugars) was determined by a modification of the method described by Jennings and Morton (1963). Flour was hydrolysed with 1.0 N HCl for 6 h in a boiling water bath. After clarification of the hydrolysate by filtration, the carbohydrate content was determined by the phenol method of Dubois *et al* (1956). Control hydrolysates of starch were included in each batch of determinations.

### **$\alpha$ -Amylase assay**

$\alpha$ -Amylase was extracted from 0.1-g flour samples by mixing for 5 min in 1 ml 0.1 M NaCl and 0.004 M  $\text{CaCl}_2$ . Aliquots of the clarified extracts were diluted twofold with 0.1 M citric acid-sodium citrate buffer (pH 5.0) or 0.1 M Tris-HCl buffer (pH 7.1) or 0.1 M Tris-HCl buffer (pH 8.8).  $\alpha$ -Amylase was determined at these three pH values by the method of McCleary and Sheehan (1987) using a Biocon Ceralpha test kit (Biocon Australia Pty Ltd, Boronia).

### **Sodium dodecyl sulphate- (SDS) sedimentation test**

Wholemeals were tested by the rapid SDS-sedimentation test of Cressey and McStay (1987b) except that the test was performed in 25-ml measuring cylinders and all ingredients were reduced fivefold. Sediment volumes were recorded after 15 min settling time.

### **SDS-polyacrylamide gel electrophoresis (SDS-PAGE)**

Single grains were crushed and either directly extracted with an SDS and mercaptoethanol extraction mixture or incubated at 37°C for 1.5 h in 0.05 M glycine-NaOH buffer (pH 9.0) before extraction as described by Cressey and McStay (1987a). Total extracted protein was separated on 17% polyacrylamide gel according to a modification (Payne *et al* 1980) of the method of Laemmli (1970). The gel was stained with Coomassie brilliant blue R250 by the method of Wyckoff *et al* (1977).

### **Germination test**

After soaking wheat for 2 h in distilled water, 100 kernels were placed on moist filter paper in a Petri dish and kept at 20°C away from light. The proportion (%) of wheat germinated was recorded at 7 days.

## **RESULTS AND DISCUSSION**

### **Visible damage, yield loss, germination and grain development**

Table 1 shows visible damage increased with earlier colonisation in Rongotea, but not consistently in Karamu. Many of the kernels infested at late flowering had the pale areas characteristic of bug damage but most kernels were so badly discoloured and shrivelled or distorted at the damage site that the insect puncture marks were often difficult to identify. Only a small proportion of kernels infested at the watery ripe stage were shrivelled. Most kernels only had insect puncture marks surrounded by pale areas and usually with slight distortions of the kernel at the site of injury. Most kernels infested at the late milky ripe stage only had insect puncture marks surrounded by pale areas; only a small proportion of kernels were distorted at the site of injury and no kernels were severely shrivelled.

The growth stage at which the commercial lines of wheat were infested was unknown, but comparison of the visual appearance of these samples with the *Nysius*-infested grain described above indicates that the bug-damaged Oroua was

TABLE I  
Properties of wheat infested by *Nysius huttoni* at various wheat growth stages

| Wheat growth stage<br>at infestation                   | Rongotea |      |      | Karamu |         |      |      |      |      |
|--|----------|------|------|--------|---------|------|------|------|------|
|  | Control  | GS69 | GS71 | GS77   | Control | GS69 | GS71 | GS77 | SEM  |
| Damaged kernels (%) <sup>a</sup>                       | 0        | 99   | 95   | 92     | 0       | 96   | 84   | 92   |      |
| Puncture marks/100 kernels <sup>b</sup>                | 0        | NM   | 206  | 155    | 0       | NM   | 311  | 272  |      |
| Screenings (%) <sup>a</sup>                            | 0.7      | 27.8 | 5.2  | 3.9    | 1.7     | 30.7 | 13.6 | 4.2  |      |
| Kernel weight (mg per kernel) <sup>a</sup>             | 50.1     | 35.4 | 42.0 | 47.0   | 39.3    | 32.4 | 34.7 | 39.0 |      |
| Germination (%) <sup>c</sup>                           | 98       | 72   | 99   | 97     | 100     | 99   | 100  | 99   | 1    |
| Total protein (%) <sup>c</sup>                         | 14.4     | 18.6 | 16.0 | 14.9   | 12.0    | 14.2 | 12.8 | 12.2 | 0.1  |
| Water soluble protein (%) <sup>c</sup>                 | 1.2      | 1.3  | 1.2  | 1.1    | 0.9     | 1.0  | 0.9  | 1.0  | 0.1  |
| Non-protein N (μEq N/g meal) <sup>c</sup>              | 8.8      | 16.5 | 8.1  | 7.6    | 8.9     | 11.1 | 7.3  | 7.8  | 0.3  |
| Total carbohydrate (%) <sup>c</sup>                    | 78.0     | 59.5 | 72.0 | 75.5   | 77.0    | 60.5 | 64.5 | 70.0 | 2.7  |
| α-Amylase (U g <sup>-1</sup> meal) <sup>c</sup> pH 5.0 | 0.16     | 4.00 | 0.20 | 0.17   | 0.17    | 1.86 | 0.18 | 0.16 | 0.03 |
| pH 7.1   | 0.09     | 1.19 | 0.12 | 0.10   | 0.09    | 0.66 | 0.13 | 0.09 | 0.03 |
| pH 8.8   | 0        | 0.09 | 0    | 0      | 0       | 0.06 | 0    | 0    |      |
| SDS-sediment,<br>non-incubated (ml) <sup>c</sup>       | 11.4     | 6.8  | 7.1  | 7.2    | 11.0    | 7.6  | 7.5  | 7.5  | 0.2  |
| SDS-sediment,<br>incubated (ml) <sup>c</sup>           | 7.5      | 3.2  | 2.7  | 2.5    | 7.7     | 2.3  | 2.5  | 2.4  | 0.2  |

<sup>a</sup> The total sample was analysed.  
<sup>b</sup> NM = Not measured; most kernels were shrivelled and insect puncture marks were difficult to define.  
<sup>c</sup> Means of duplicate determinations are presented; standard error of mean (SEM) calculated for each data set is shown. Calculations included data from Table 2.

infested at the watery ripe stage and the bug-damaged Kopara was infested at the milky ripe stage. Commercial lines of wheat infested earlier than the watery ripe stage of grain development would be so badly shrivelled that the grain would be classed as feed wheat and would not be sent to WRI for bake testing.

The effect of *Eurygaster* and *Aelia* (Sunn pests) on grain appearance seems to be more severe than that of *Nysius*. Tischler (1939) showed that grain infested before the milky ripe growth stage became severely shrivelled whereas kernels infested during the milky ripe stage were partly shrivelled and partly dented with pierce marks surrounded by pale areas. Kernels infested during the ripening stage (late maturity) only had pierce marks surrounded by pale areas. Paulian and Popov (1980) reported that grain damaged by the Sunn pest during the milky ripe stage was completely destroyed and even kernels infested in late wheat maturity were slightly shrivelled.

A marked grain yield loss through an increase in the proportion of screenings and reduced kernel weight was detected in wheat colonised with *Nysius* at the late flowering stage (Table 1). A further yield fraction may be lost due to complete abortion of the developing seed, as found in Mirid damage to lucerne (Farrell J A K and Stufkens M W, unpublished data). In studies carried out on a large number of wheat samples exhibiting different degrees of attack by the Sunn pest in Europe, the kernel weight of damaged grain was reduced to 78.3–92.4% of that of undamaged grain (Paulian and Popov 1980). In contrast, New Zealand wheat heavily infested with *Nysius* during the milky stage had kernel weights reduced to 94–99% of that of undamaged grain (Table 1).

Reduced germination capacity only occurred for *Rongotea* infested at the late flowering stage (Table 1). The low germination capacity of bug-damaged Oroua (Table 2) could be the effect of insect infestation of grain at a very immature stage or the effect of storing the grain for four years at 4°C. Tischler (1939) found that grain infested by the Sunn bug after the milky ripe stage had undiminished germination capacity. However, studies on a large number of bug-damaged wheat samples in Europe showed that samples containing as little as 10% damaged kernels had germination capacity reduced below accepted limits (Paulian and Popov 1980). Infestation by *Eurygaster* reduced germination capacity much more than infestation by *Aelia* (Tischler 1939). Meneret (1936) observed that grain damaged near the germ by Sunn bugs had half the germination capacity of kernels damaged at other sites.

### Enzyme activity and grain development

All the samples infested by *Nysius* had strong wheat bug proteinase activity as shown by the incubated SDS sedimentation test (Table 1) and the disappearance of high molecular weight (HMW) glutenin subunits from the electrophoretogram (Fig 1). These properties were similar to those demonstrated for the commercial lines of bug-damaged wheat (Cressey and McStay 1987a).

In contrast, wheat heavily infested by *Nysius* during the watery ripe and milky ripe stages had low  $\alpha$ -amylase activity similar to control wheat when measured at acid, neutral or alkaline pH values (Table 1). Thus *Nysius* does not inject stable  $\alpha$ -amylase activity into grain while feeding. The high  $\alpha$ -amylase activity in both



**Fig 1.** SDS-PAGE electrophoretogram of total protein from incubated single kernels. Protein subunits from Rongotea are in lanes (from the left) 1 to 4 and Karamu in lanes 5 to 8. Lanes 1 and 5 contain protein from control wheat; lanes 2 and 6 wheat infested at GS69; lanes 3 and 7 wheat infested at GS71; and lanes 4 and 8 wheat infested at GS77. The HMW subunits are the distinct group of five bands at the top of the gel.

cultivars infested by *Nysius* at the late flowering stage of wheat development was probably endogenous wheat enzyme because of the maximum activity at pH 5 (Table 1). Insect salivary  $\alpha$ -amylases are usually most active at pH 6 to 7 (Nuorteva 1954; Hori 1972; Dexter *et al* 1987). Lorenz and Meredith (1988) showed that starch granules were pitted near the site of *Nysius* bite marks in grain colonised at late flowering. They interpreted this as evidence of  $\alpha$ -amylase activity but could not measure enzyme activity by the Falling Number test.

At the late flowering stage, wheat has a high level of  $\alpha$ -amylase which usually declines during the milk development stage (Marchylo *et al* 1980; Meredith 1982; Gale *et al* 1983). It is possible that the severe disruption of physiological development of the grain by *Nysius* infestation at late flowering destroys the mechanism by which  $\alpha$ -amylase decays. A similar effect seems to occur when some diseases, aphids or frosts damage immature grain (Rautapää 1968; Meredith 1977; Meredith and Pomeranz 1985). It is also possible that the effect of *Nysius* on grain development predisposes the matured grain to produce  $\alpha$ -amylase similar to that in preharvest sprouted grain. *Fusarium* infection of maize can cause preharvest germination, and mould can stimulate grain to produce amylases before harvest (Meredith and Pomeranz 1985).

The commercial line of bug-damaged Aotea had higher than normal  $\alpha$ -amylase activity at pH 5 (Table 2). This might indicate that the wheat was infested at an early stage of development to cause curtailed development or susceptibility to preharvest sprouting. Alternatively, this sample may have been infested by an insect such as *Lincolnia lucernina* (native to New Zealand) which appears to inject salivary  $\alpha$ -amylase into wheat while feeding. Caged wheat infested by *L. lucernina* at the watery ripe stage had very high  $\alpha$ -amylase activity at an optimum of pH 5.5, and the

TABLE 2  
Properties of commercial lines of bug-damaged wheat

|  | <i>Aotea</i> <sup>a</sup> | <i>Oroua</i> | <i>Kopara</i> |
|--|---------------------------|--------------|---------------|
| Damaged kernels (%)                                | —                         | 29           | 24            |
| Puncture marks per 100 kernels                     | —                         | 34           | 29            |
| Kernel weight (mg per kernel)                      | —                         | 36.5         | 37.9          |
| Germination (%)                                    | —                         | 87           | 95            |
| Total protein (%)                                  | 10.3                      | 13.8         | 9.9           |
| Non-protein N ( $\mu$ Eq N g <sup>-1</sup> flour)  | 15.5                      | 26.2         | 8.2           |
| Total carbohydrate (%)                             | 77.0                      | 68.5         | 78.5          |
| $\alpha$ -Amylase (U g <sup>-1</sup> flour) pH 5.0 | 0.46                      | 0.26         | 0.12          |
| pH 7.1   | 0.04                      | 0.11         | 0.06          |
| pH 8.8   | 0                         | 0            | 0             |

<sup>a</sup> White flour was analysed for *Aotea* and wholemeal flour was analysed for *Oroua* and *Kopara*.

endosperm starch granules were almost completely missing in a large area surrounding the site of insect attack (Every D, Farrell J A K, Stufkens M W, unpublished). The low  $\alpha$ -amylase activity of the other two commercial lines of bug-damaged wheat (Table 2) might indicate that they were infested by *Nysius* during the milk development stage of wheat.

In Europe wheat infested by the Sunn pests always has high protease activity and sometimes higher than normal  $\alpha$ -amylase activity (Kretovich 1944; Yakovenko *et al* 1973). Wheat injured by *Lygus nugulipennis* contains high  $\alpha$ -amylase activity and normal protease activity but still bakes normally (Nuorteva 1954). These studies and the results of this paper and Every *et al* (1989) demonstrate that only high bug protease activity in wheat causes the characteristic bug damage problem in baking; bug salivary  $\alpha$ -amylase is not involved.

### Biochemical changes and grain development

When compared with uninfested control wheat, wheat infested by *Nysius* at late flowering had high total protein, normal water-soluble protein (albumins), high non-protein nitrogen (free amino acids) and low carbohydrate content (Table 1). Wheat infested at the watery ripe stage was less affected, and wheat infested at the milky ripe stage was little changed from control wheat. This indicates that *Nysius* infestation of wheat at a growth stage before or near the beginning of grain-filling severely affects protein and/or carbohydrate metabolism.

Normally, starch synthesis and accumulation commence about 5 to 10 days post-anthesis, just before the appearance of storage protein (Bilinski and McConnell 1958; Jennings and Morton 1963; Donovan *et al* 1977a,b). While these components accumulate, the absolute amount (mg per grain) of free amino acids and sugars remains reasonably constant (Jennings and Morton 1963) or ultimately declines (Donovan *et al* 1977a) throughout development. Donovan *et al* showed that a wheat cultivar with high protein had higher free amino acid content and lower kernel weight than a cultivar with low protein. They suggested that the higher levels

of free amino acids reaching the grain by translocation from other parts of the plant may lead to the higher rate of grain protein accumulation, and hence to a higher final grain protein content, provided the duration of synthesis is maintained. Similarly, high protein is associated with high free amino acid content and low kernel weight in wheat infested by *Nysius* at late flowering. However, the high protein content may not be a consequence of a higher rate of protein synthesis, but may result from a reduced rate of starch synthesis and accumulation. This suggestion is consistent with the low carbohydrate content and the reduced endosperm content of the shrivelled grain. The high  $\alpha$ -amylase activity in grain infested at late flowering might also have contributed to the low carbohydrate content by causing degradation of starch.

These effects are similar to those caused by moisture stress during the critical stages of grain-filling, where disrupted protein and starch metabolism produced shrivelled kernels of higher than usual protein content (Simmonds and O'Brien 1981). Starch metabolism in developing grain is also disrupted by thermal stress (30°C) at any time during anthesis and the first 7 days after anthesis (MacLeod and Duffus 1988). It was shown that starch deposition was reduced as a consequence of reduced sucrose synthase activity and was not a result of limitation of assimilate (sucrose).

The pattern of biochemical changes in wheat infested by the Sunn pest in Europe is quite different from that of *Nysius*-infested wheat. The protein content is lower than normal, and the water soluble protein is over twice as high as normal (Kretovich 1944; Pokrovskaya *et al* 1971). It was not known at what stage of wheat development infestation occurred but it was assumed to be some time during the milk development stage. However, the results indicated that the biochemical changes were not caused by disrupted physiological development but were caused by insect salivary proteases degrading gluten to water or alcohol soluble protein fragments (Kretovich 1944). In New Zealand bug-damaged wheat the insect proteinase degrades gluten to acetic acid or SDS-soluble protein fragments, but not water or alcohol soluble degradation products (Cressey 1987a; Cressey and McStay 1987a).

### Feeding mechanism and grain development

Three possible feeding mechanisms have been suggested for heteropterous insects (bugs). The first suggestion is that a bug simply pierces the immature grain with its stylus and sucks out the milky succulent juices (Blair and Morrison 1949; Doss 1980). However, it is not known whether the endosperm protein and carbohydrate content of wheat at the milky development stage is sufficiently fluid to be sucked through the bug stylus.

The second method assumes that a bug injects salivary juices containing powerful enzymes which digest the grain carbohydrate and/or protein so that solubilised nutrients can be drawn through the bug stylus (Kretovich 1944; Meredith 1970; Doss 1980; Cressey and McStay 1987b). There is evidence that this occurs for Sunn pests in Europe (Kretovich 1944; Hinton 1962) and *Lincolnia lucernina* in New Zealand (Every D, Farrell J A K, Stufkens M W, unpublished). Doss (1980) states that *Aelia germari* Kust feeds by this method only on fully mature dry grain.

In New Zealand, the insects that caused bug damage in two commercial lines of wheat and *Nysius* do not inject  $\alpha$ -amylase into wheat, and the salivary protease which is present in the grain does not appear to digest gluten to water soluble nutrients for easy assimilation by the insects (Cressey 1987b; Tables 1 and 2). Other heteropterous insects in New Zealand can feed on wheat without injection of  $\alpha$ -amylase and protease into the grain, although they produce similar feeding marks on the cheeks of kernels as those produced by the Sunn pest and *Nysius*. These bugs are *Calocoris norvegicus*, *Sidnia kinbergi* (Cressey *et al* 1987; Lorenz and Meredith 1988), *Dictyotus caenosus* and *Rhyodes* sp (Every D, Farrell J A K, Stufkens M W, unpublished). Nuorteva (1954) suggested that, because of the short feeding times and low salivary enzyme activities in certain bugs, hardly any extra-intestinal digestion of plant material can occur and any salivary enzymes are used chiefly in the insects' alimentary tract.

The third possible bug feeding mechanism (Jenner C F pers comm) explains how these bugs might extract nutrients from wheat without prior digestion by salivary enzymes. The bugs could suck sap rich in amino acids and sugars from two lateral protophloem strands in the ovary at late anthesis or two lateral sieve tubes in young grain during the milky development stage. The purpose of bug saliva injected into the grain may be to help maintain the flow of sieve tube sap, that is, sieve tube bleeding. The salivary juices may also soften the husks and outer pericarp of wheat to facilitate bug stylus penetration. Some heteropterous insects have a salivary polygalacturonase which degrades the plant cell walls and assists stylus penetration (Laurema *et al* 1985). These suggestions arise from observations on the vascular system of immature grain in relation to aphid feeding behaviour (Fisher and Gifford 1986). The idea is consistent with the localised site of wheat-bug feeding marks on bug-damaged kernels. For all the *Nysius*-infested samples and commercial lines of bug-damaged wheat in this study, 96–100% of bug feeding marks were located on the lateral surface, or cheek, of the grain. A few puncture marks were located on the ventral surface of the grain and none on the dorsal surface. Some multiply injured kernels had a clear line of puncture marks along the lateral surface (Fig 2). The pattern of *Nysius* feeding marks was very similar to that of *Lygus gugulipennis*



Fig. 2. Rongotea kernel multiply injured by *Nysius* in a line along the lateral surface

(Nuorteva 1956) which threads its mouthparts between the husks. Insects which feed by inserting mouthparts through the husk (*Dolycoris baccarum*) or direct the stylus into the kernel from above (*Miris dolabratus*) produce a distinctly different pattern of feed marks on kernels (Nuorteva 1956).

If *Nysius* does suck sap from the vascular bundles of immature grain, then the probable interference with nutrient transport to developing ovaries in late anthesis could lead to the observed disruption of morphological and physiological development of bug-damaged wheat. Restriction of nutrients at this critical stage just before or at the initiation of synthesis of endosperm storage proteins and carbohydrates appears to disrupt these processes and drastically affect grain-filling.

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### REFERENCES

- Association of Official Analytical Chemists 1981 *Changes in Methods. Supplement to 13th Edition*, Vol 64, No 2, ed Horwitz W. Methods 24.B01 and 24.B03. AOAC, Washington, DC.
- Bradford M M 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Anal Biochem* **72** 248–254.
- Bilinski E, McConnell W B 1958 Studies on wheat plants using carbon-14 compounds. VI Some observations of protein biosynthesis. *Cereal Chem* **35** 66–81.
- Blair I D, Morrison L 1949 Wheat diseases and insect pests. *Information Series No 3*. Department of Scientific and Industrial Research, Wellington.
- Cressey P J 1987a Studies on bug damage in New Zealand wheats. *Wheat Research Institute Report No WRI 109*. New Zealand Department of Scientific and Industrial Research, Christchurch, pp 1–21.
- Cressey P J 1987b Wheat-bug damage in New Zealand wheats: some properties of a glutenin hydrolysing enzyme in bug-damaged wheat. *J Sci Food Agric* **41** 159–165.
- Cressey P J, McStay C L 1987a Wheat-bug damage in New Zealand wheats. Development of a simple SDS sedimentation test for bug damage. *J Sci Food Agric* **38** 357–366.
- Cressey P J, McStay C L 1987b A rapid SDS sedimentation test for detection of bug damage to wheat. *Wheat Research Institute Report No WRI 108*. New Zealand Department of Scientific and Industrial Research, Christchurch, pp 1–14.
- Cressey P J, Farrell J A K, Stufkens M W 1987 Identification of an insect species responsible for bug damage in New Zealand wheats. *NZ J Agric Res* **30** 209–212.
- Dexter J E, Preston K R, Cooke L A, Morgan B C, Kruger J E, Kilborn R H, Elliott R H 1987 The influence of orange wheat blossom midge (*Sitodiplosis mosellana* Géhin) damage on hard red spring wheat quality and the effectiveness of insecticide treatments. *Can J Plant Sci* **67** 697–712.
- Donovan G R, Lee J W, Hill R D 1977a Compositional changes in the developing grain of high- and low-protein wheat. I Chemical composition. *Cereal Chem* **54** 638–645.

- Donovan G R, Lee J W, Hill R D 1977b Compositional changes in the developing grain of high- and low-protein wheat. II Starch and protein synthetic capacity. *Cereal Chem* **54** 646–656.
- Doss S A 1980 Note on the biology of wheat bug, *Aelia germari* Kust (Heteroptera: Pentatomidae), in Algeria. *Indian J Agric Sci* **50** 372–374.
- Dubois M, Gilles K A, Hamilton J K, Rebers P A, Smith F 1956 Colorimetric method for the determination of sugars and related substances. *Anal Chem* **28** 350–356.
- Every D, Farrell J A K, Stufkens M W 1989 Effect of *Nysius huttoni* on the protein and baking properties of two New Zealand wheat cultivars. *NZ J Crop Hort Sci* **17** 7–12.
- Fisher D B, Gifford R M 1986 Accumulation and conversion of sugars by developing wheat grains. VI Gradients along the transport pathway from the peduncle to the endosperm cavity during grain filling. *Plant Physiol* **82** 1024–1030.
- Gale M D, Flintham J E, Arthur E D 1983 Alpha-amylase production in the late stages of grain development—an early sprouting damage risk period? In: *Third Int Symp Pre-harvest Sprouting in Cereals*, eds Kruger J E & La Berge D E. Westview Press, Boulder, Colo, pp 29–35.
- Hanford J 1967 The proteolytic enzymes of wheat and flour and their effect on bread quality in the United Kingdom. *Cereal Chem* **44** 499–511.
- Hinton J J C 1962 A description of bug-damaged wheat. *Res Assoc Br Flour-millers' Bull* **13** 16–20.
- Hori K 1972 Comparative study of a property of salivary amylase among various heteropterous insects. *Comp Biochem Physiol* **42B** 501–508.
- Jennings A C, Morton R K 1963 Changes in carbohydrate, protein, and non-protein nitrogenous compounds of developing wheat grain. *Aust J Biol Sci* **16** 319–331.
- Kent-Jones D W, Amos A J 1938 The wheat bug. *Food* December 1–8.
- Kretovich V L 1944 Biochemistry of the damage to grain by the wheat-bug. *Cereal Chem* **21** 1–16.
- Laemmli U K 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227** 680–685.
- Laurema S, Varis A L, Miettinen H 1985 Studies on enzymes in the salivary glands of *Lygus rugulipennis* (Hemiptera, Miridae). *Insect Biochem* **15** 211–244.
- Lorenz K, Meredith P 1988 Insect-damaged wheat—effects on starch characteristics. *Starch* **40** 136–139.
- MacLeod L C, Duffus C M 1988 Reduced starch content and sucrose synthase activity in developing endosperm of barley plants grown at elevated temperatures. *Aust J Plant Physiol* **15** 367–375.
- McCleary B V, Sheehan H 1987 Measurement of cereal  $\alpha$ -amylase: a new assay procedure. *J Cereal Sci* **6** 237–251.
- Marchylo B A, Lacroix L J, Kruger J E 1980  $\alpha$ -Amylase isoenzymes in Canadian wheat cultivars during kernel growth and maturation. *Can J Plant Sci* **60** 433–443.
- Meneret G 1936 Influence des piqûres de punaises sur la qualité des bles. *Le selectionneur* **5** 1–12.
- Meredith P 1970 'Bug' damage in wheat. *NZ Wheat Rev* **11** 49–53.
- Meredith P 1977 Amylase activities in frosted wheat. *NZ J Sci* **20** 465–467.
- Meredith P 1982 Aspects of field-sprouting of wheat. In: *Developments in Food Science 5A Progress in Cereal Chemistry and Technology. Part A. Proceedings of the 7th World Cereal and Bread Congress*, Prague, eds Holas J & Kratochvil J. Elsevier Science Publishers, Amsterdam, pp 127–132.
- Meredith P, Pomeranz Y 1985 Sprouted grain. In: *Advances in Cereal Science and Technology 7*, ed Pomeranz Y. American Association of Cereal Chemists, Minneapolis, Minn, pp 239–320.
- Morrison I 1938 Surveys of the insect pests of wheat crops in Canterbury and North Otago during the summers of 1936–37 and 1937–38. *NZ J Sci Technol* **20** 142–155.
- Nuorteva P 1954 Studies on the salivary enzymes of some bugs injuring wheat kernels. *Ann Entomol Fennici* **20** 102–124.

- Nuorteva P 1956 The possibility of distinguishing the symptoms of injury to wheat kernels made by different heteropterous bugs. *Ann Entomol Fennici* **22** 120–121.
- Paulian F, Popov C 1980 Sunn pest or cereal bug. In: *Wheat*, ed Hafliger E. Ciba-Geigy, Basle, pp 69–74.
- Payne P I, Law C N, Mudd E E 1980 Control of the homologous group 1 chromosomes of the high-molecular-weight subunits of glutenin, a major protein in wheat endosperm. *Theor Appl Genet* **58** 113–120.
- Pokrovskaya N F, Morozova G I, Vinogradova N M 1971 Proteins of wheat grain damaged by the shell-bug *Eurygaster intergriceps* Put. *Prikl Biokhim I Mikrobiol* **7** 121–127.
- Rautapää J 1968 Reduction in yield and changes in brewing quality of barley caused by *Macrosiphum avenae* (F.) (Hom., Aphididae). *Acta Agric Scand* **18** 233–241.
- Simmonds D H, O'Brien T P 1981 Morphological and biochemical development of the wheat endosperm. *Adv Cereal Sci Technol* **4** 5–70.
- Tischler W 1939 Schaden und Bekämpfung der getreideschadlichen Blattwanzen. *Arbeit Physiol Angew Entomol Berl Dahlem* **6** 14–32.
- Wyckoff M, Rodbard D, Chrambach A 1977 Polyacrylamide gel electrophoresis in sodium dodecyl sulfate-containing buffers using multiphasic buffer systems: properties of the stock, valid  $R_f$ -measurement, and optimized procedure. *Anal Biochem* **78** 459–482.
- Yakovenko V A, Litvinov A M, Stoyanova A A 1973 Characteristics of gluten protein of wheat attacked by the wheat bug. *Izv Vyss Uchebn Zaved Pishch Tekhnol* No 4 17–19.
- Yemm E W, Cocking E C 1955 The determination of amino-acids with ninhydrin. *Analyst (London)* **80** 209–213.
- Zadoks J C, Chang T T, Konzak C F 1974 A decimal code for the growth stages of cereals. *Weed Res* **14** 415–421.



## Influence of Nitrogen Form and Concentration on the Nitrate Reductase Activity of Winter Barley (*Hordeum vulgare* L cv Igri)

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### ABSTRACT

Winter barley (*Hordeum vulgare* L cv Igri) plants were grown for 5 weeks in a controlled environment room using a recirculating nutrient solution culture system at a root and shoot temperature of 10°C.

When solution  $\text{NO}_3\text{-N}$  concentration was varied between 0 and 64 mg litre<sup>-1</sup>, shoot endogenous and induced nitrate reductase activity ( $\text{NRA}_e$  and  $\text{NRA}_i$  respectively) increased asymptotically from zero in plants grown with 0 mg  $\text{NO}_3\text{-N}$  litre<sup>-1</sup> to a maximum activity in solutions containing  $\geq 16$  mg  $\text{NO}_3\text{-N}$  litre<sup>-1</sup>. Shoot Nitrogen Response Index ( $\text{NRI} = \text{NRA}_i / \text{NRA}_e$ ) showed an opposite trend decreasing from a maximum of 2.6 at 2 mg  $\text{NO}_3\text{-N}$  litre<sup>-1</sup> to a minimum approaching unity when  $\geq 32$  mg N litre<sup>-1</sup> was supplied.

In a separate experiment, nitrogen form ( $\text{NO}_3\text{-N}$ ,  $\text{NH}_4\text{-N}$  or a 1:1 mixture) had a marked influence on shoot NRA. Enzyme activity was negligible in tissue from plants absorbing  $\text{NH}_4\text{-N}$ , highest in  $\text{NO}_3\text{-N}$ -fed plants and intermediate in those supplied with both N forms. NRI, however, approached unity in all treatments.

The results provide evidence to suggest that NRI may be used as an indicator of the N status of barley plants grown with varied N nutrition.

**Key words:** Nitrate reductase activity, Nitrogen Response Index, nitrate, ammonium, winter barley.

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## INTRODUCTION

Farmers in the UK apply around 1.3 million tonnes of fertiliser N annually, of which cereals receive one-third (Elsmere 1988). However, results from field and lysimeter experiments have shown that arable crops recover on average only 45 to 50% of applied N; the remainder is retained in soil organic matter/biomass or lost from the soil by denitrification and/or leaching (Allison 1966; Dowdell 1982).

Crop requirements for fertiliser N depend on the quantity of soil organic N mineralised during the growing season. Up to now, attempts to develop simple, rapid, reliable tests to determine potentially mineralisable N in soils have failed (Stanford 1982). Numerous other attempts have been made to predict crop fertiliser N requirements. The method used by UK advisory services is based on a knowledge of previous cropping to estimate soil N status. Fertiliser N recommendations are then modified according to N status, soil type and other environmental factors (MAFF 1983; SAC 1985). Despite its widespread use, the system is acknowledged as imprecise and therefore inefficient (Jenkinson 1984).

Predictive models (Greenwood 1982) and analysis of the growing crop for total N and  $\text{NO}_3\text{-N}$  (Verstraeten and Vlassak 1981) have also been used to estimate fertiliser N requirement. All these methods have drawbacks. For example, total N in shoot material is relatively insensitive to changes in N supply (Bar-Akiva 1970; Papastylianou 1986), and tissue nitrate concentration does not reflect soil N availability when plants are absorbing a significant proportion of their N in ammonium form.

Using nitrate reductase activity (NRA) as a measure of plant N status overcomes some of these problems. This substrate-inducible enzyme has been shown to be a sensitive and responsive measure of plant N status and hence to reflect N availability at the root at the time of sampling (Bar-Akiva *et al* 1970). However, this method has not been generally accepted, due partly to the complexity of plant and environmental factors known to influence NRA, and partly to the lack of awareness of the need to optimise the assay technique for the specific crop being grown (Sylvester-Bradley *et al* 1984).

A more thorough appraisal than hitherto is required to examine fully the potential of NRA to assess crop N status, soil N status and, ultimately, fertiliser N requirement. An earlier paper by one of the authors (Sym 1984) detailed the first step in such an appraisal related to winter barley: the optimisation of a precise, reliable method to determine NRA. The present paper describes the next stage of this work: a study of the influence of nitrogen nutrition on the ability of NRA measurements to predict the N status of winter barley plants grown under simulated conditions of late winter/early spring.

## METHODS

### Culture system

Experiments were conducted in a controlled environment room using a recirculating nutrient solution system. Three 150-litre nutrient solution reservoirs

each supplied six culture trays ( $40 \times 25 \times 8$  cm) fitted with overflows which drained back to the reservoirs. Solution depth within each tray was maintained at 6 cm with a flow rate of  $0.7 \text{ litre min}^{-1}$ . The 18 trays (six replicates of each of the three treatments) were arranged in a randomised block design on a bench. The three reservoirs were fitted with thermostats and heating/cooling coils for solution temperature control.

Chitted winter barley seeds (*Hordeum vulgare* L cv 'Igri') were prepared by soaking in aerated water for 5 h before being germinated on moist tissue paper for 40 h in the dark at room temperature. Twenty chitted seeds were then sown in 13-cm-diameter pots containing perlite. Four pots per tray were placed on 3-cm-thick polystyrene blocks which allowed a 3-cm solution depth to be maintained in the lower layers of the perlite.

During the first five days after sowing, all plants were grown at a root and shoot temperature of  $30 \pm 1^\circ\text{C}$  in a nitrogen-free solution containing the following nutrients ( $\text{mg litre}^{-1}$ ): K 320, Ca 160, Mg 40, P 40, Fe 4, Mn 2, B 0.8, Zn 0.4, Co 0.4, Mo 0.08. Solution pH was 6.3 and conductivity  $1500 \mu\text{S cm}^{-1}$ . Light intensity at the pot surface was 7 klx with a 12-h photoperiod. On the sixth day, seedlings were thinned to leave 15 plants per pot (60 plants per replicated treatment), and shoot and root temperatures were reduced step-wise to achieve  $10 \pm 1^\circ\text{C}$  on the eleventh day after sowing when the three nutrient solution treatments were started.

#### **Experiment 1. The influence of nitrate concentration on the growth, N status and NRA of barley plants**

This work was conducted over three separate time intervals, corresponding to Experiments 1A, 1B and 1C;  $\text{NO}_3^-$ -N concentration of the recirculating nutrient solutions varied from 0 to  $64 \text{ mg litre}^{-1}$ . Experiment 1A used 0, 2 ( $\pm 0.5$ ) and 4 ( $\pm 0.8$ )  $\text{mg NO}_3^-$ -N  $\text{litre}^{-1}$ ; Experiment 1B: ( $\pm 0.8$ ), 8 ( $\pm 1$ ) and 16 ( $\pm 1$ )  $\text{mg NO}_3^-$ -N  $\text{litre}^{-1}$ ; Experiment 1C: 16 ( $\pm 1$ ), 32 ( $\pm 2$ ) and 64 ( $\pm 4$ )  $\text{mg NO}_3^-$ -N  $\text{litre}^{-1}$ . Other environmental conditions were kept constant as described previously. The recirculating nutrient solutions were analysed every two days for  $\text{NO}_3^-$ -N (phenol disulphonic acid method; Jackson 1958) and adjusted as required using calcium nitrate solution to maintain the desired  $\text{NO}_3^-$ -N concentrations. Solution pH was kept within the range 6.3–6.8 by adding dilute sulphuric acid or calcium hydroxide solution. N-free nutrient stock solution was used to control conductivity between 1800 and  $2200 \mu\text{S cm}^{-1}$ .

#### **Experiment 2. The influence of nitrogen form on the growth, N status and NRA of barley plants**

This experiment was carried out over a single time interval. Three complete nutrient solutions were prepared containing  $20 \text{ mg N litre}^{-1}$  as either 100%  $\text{NO}_3^-$ -N, 100%  $\text{NH}_4^+$ -N or a 1:1 mixture of  $\text{NH}_4^+$ -N and  $\text{NO}_3^-$ -N. Other environmental conditions were identical to those in Experiment 1. The  $\text{NO}_3^-$ -N content of the recirculating nutrient solutions was checked and adjusted regularly as described previously.  $\text{NH}_4^+$ -N concentration was determined by Nessler's method (Jackson 1958) and adjusted using ammonium sulphate solution. Nutrient solution pH and conductivity were maintained as described for Experiment 1.

## Plant analysis

Shoots of all plants in both Experiments 1 and 2 were harvested at the fourth leaf stage and fresh weights were recorded. Fresh material was chopped into 1-cm lengths, bulked, sub-sampled and assayed for in-vivo endogenous ( $\text{NRA}_e$ ) and induced ( $\text{NRA}_i$ ) nitrate reductase activity (Sym 1984).  $\text{NRA}_e$  is a measure of active tissue enzyme determined by incubating in a medium without added nitrate;  $\text{NRA}_i$  indicates the potential enzyme activity, ie when nitrate is not limiting, and is assayed in an incubation medium containing added nitrate (Bar-Akiva *et al* 1970). The quotient  $\text{NRA}_i/\text{NRA}_e$  is defined as the Nitrogen Response Index (NRI).

The remainder of the fresh material was dried, finely milled and analysed for reduced N (using a semi-micro Kjeldahl technique). Nitrate was determined in cold water extracts by the phenol disulphonic acid method. Total N was expressed as the sum of reduced-N +  $\text{NO}_3^-$ -N.

## RESULTS AND DISCUSSION

### Experiment 1. The influence of nitrate concentration on the growth, N status and NRA of barley plants

#### *Yield, total N and $\text{NO}_3^-$ -N concentrations of dry matter*

Yield, total N and nitrate concentration of shoot dry matter increased from a minimum when no nitrate was supplied to a maximum at  $\geq 32 \text{ mg NO}_3^- \text{-N litre}^{-1}$  (Table 1). Plants grown with 0 and  $2 \text{ mg NO}_3^- \text{-N litre}^{-1}$  showed severe N deficiency symptoms.

These observations suggest that plant growth was increasingly limited by sub-optimal rates of nitrate absorption as solution nitrate-N concentration decreased from  $32$  to  $0 \text{ mg litre}^{-1}$ . This conclusion is supported by the fact that the concentrations of other nutrients were common to all treatments, and target nitrate concentrations were successfully maintained in all treatments. Even in the  $2 \text{ mg N litre}^{-1}$  treatment, the concentration in the uppermost layers of perlite never fell below  $1.4 \text{ mg N litre}^{-1}$ . Furthermore, the quantities of nitrate added to maintain solution concentrations during the growth period were directly proportional to target concentration (see Table 1).

These findings appear to contradict those for ryegrass (Clement *et al* 1974) and for tomatoes (Massey and Winsor 1980). For example, when external nitrate was maintained constant over a 1000-fold concentration range ( $0.2$  to  $200 \text{ mg NO}_3^- \text{-N litre}^{-1}$ ), there was little effect on relative growth rate, total N and  $\text{NO}_3^-$  concentration in ryegrass (Clement *et al* 1974). However, these earlier experiments were conducted at relatively high nutrient solution temperatures compared with the present one. It is well known that nitrate uptake is markedly restricted by low root temperatures. When solution temperature decreased from  $20^\circ \text{C}$  to  $4^\circ \text{C}$ , the rate of nitrate uptake by ryegrass fell by  $75\%$  (Clarkson and Warner 1979). It therefore seems likely that the rate of nitrate absorption at the low root temperatures used in the present work was the limiting factor to growth in solutions containing  $< 32 \text{ mg NO}_3^- \text{-N litre}^{-1}$ .

TABLE 1  
The influence of nitrate concentration on the yield, nitrogen content and nitrate reductase activity of winter barley shoots at the 4th leaf stage of growth

|  | Nitrate concentration in recirculating nutrient solution (mg N litre <sup>-1</sup> ) |                    |                   |                   |                   |                   |                   |                   |                   |  |
|--|--|--------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|--|
|  | Experiment 1A  |                    |                   | Experiment 1B     |                   |                   | Experiment 1C     |                   |                   |  |
|  | 0  | 2                  | 4                 | 4                 | 8                 | 16                | 16                | 32                | 64                |  |
| Fresh weight (g)   | 10.6 <sup>a</sup>  | 11.7 <sup>ab</sup> | 13.2 <sup>b</sup> | 13.6 <sup>a</sup> | 14.9 <sup>b</sup> | 17.2 <sup>c</sup> | 18.0 <sup>a</sup> | 21.0 <sup>b</sup> | 23.0 <sup>c</sup> |  |
| Dry weight (g)   | 1.6 <sup>a</sup>   | 1.7 <sup>a</sup>   | 1.8 <sup>a</sup>  | 1.8 <sup>a</sup>  | 1.9 <sup>ab</sup> | 2.2 <sup>b</sup>  | 2.2 <sup>a</sup>  | 2.5 <sup>b</sup>  | 2.6 <sup>b</sup>  |  |
| Total N (‰ DM)   | 1.9 <sup>a</sup>   | 2.6 <sup>b</sup>   | 2.9 <sup>c</sup>  | 3.4 <sup>a</sup>  | 3.4 <sup>a</sup>  | 3.6 <sup>a</sup>  | 3.7 <sup>a</sup>  | 4.0 <sup>a</sup>  | 4.0 <sup>a</sup>  |  |
| NO <sub>3</sub> <sup>-</sup> -N (‰ DM)   | 0.00 <sup>a</sup>  | 0.04 <sup>b</sup>  | 0.09 <sup>c</sup> | 0.10 <sup>a</sup> | 0.14 <sup>b</sup> | 0.17 <sup>c</sup> | 0.21 <sup>a</sup> | 0.26 <sup>b</sup> | 0.31 <sup>c</sup> |  |
| NRA <sub>e</sub> (μmol NO <sub>2</sub> <sup>-</sup> g FW <sup>-1</sup> h <sup>-1</sup> ) | 0.00 <sup>a</sup>  | 0.36 <sup>b</sup>  | 0.66 <sup>c</sup> | 0.62 <sup>a</sup> | 1.08 <sup>b</sup> | 1.44 <sup>c</sup> | 1.60 <sup>a</sup> | 1.47 <sup>a</sup> | 1.49 <sup>a</sup> |  |
| NRA <sub>i</sub> (μmol NO <sub>2</sub> <sup>-</sup> g FW <sup>-1</sup> h <sup>-1</sup> ) | 0.00 <sup>a</sup>  | 0.93 <sup>b</sup>  | 1.11 <sup>c</sup> | 1.04 <sup>a</sup> | 1.36 <sup>b</sup> | 1.59 <sup>c</sup> | 1.80 <sup>a</sup> | 1.51 <sup>b</sup> | 1.55 <sup>b</sup> |  |
| Nitrogen Response Index  | —  | 2.6 <sup>a</sup>   | 1.7 <sup>b</sup>  | 1.7 <sup>a</sup>  | 1.2 <sup>b</sup>  | 1.1 <sup>c</sup>  | 1.1 <sup>a</sup>  | 1.0 <sup>a</sup>  | 1.0 <sup>a</sup>  |  |
| Nitrate N added to maintain target concentrations during growth period (mg)              | 0  | 180                | 310               | 300               | 400               | 550               | 590               | 620               | 630               |  |

For each experiment and parameter, results followed by the same letter are not significantly different at the 5 % level by least significant difference.

### Nitrate reductase activity

Shoot  $\text{NRA}_e$  and  $\text{NRA}_i$  increased asymptotically from zero when no nitrogen was supplied to a maximum activity at  $\geq 16 \text{ mg N litre}^{-1}$  (Table 1). Shoot  $\text{NRI}$  ( $\text{NRA}_i/\text{NRA}_e$ ) showed an opposite trend, decreasing from a maximum of 2.6 when  $2 \text{ mg N litre}^{-1}$  was maintained in solution, to a minimum approaching unity when  $\geq 32 \text{ mg N litre}^{-1}$  was supplied. Since the  $\text{NRA}$  assay estimates directly the extent to which nitrate is limiting the enzyme's activity, it also measures indirectly the degree to which nitrate is limiting growth.  $\text{NRI}$  values of 1.0–1.1 indicate that growth is not limited by lack of nitrate and that plants will not respond to increases in nitrate supply. Conversely, values above unity imply that  $\text{NRA}_e$  is increasingly limited by lack of substrate and that growth will be increased by additions of nitrate. This suggests that  $\text{NRI}$  can be used not only to indicate plant  $\text{N}$  status but also to predict likely plant response to increases in external nitrate concentration. These findings agree well with the work of others who have reported similar relationships between  $\text{NRA}_i/\text{NRA}_e$  and  $\text{N}$  nutrition for citrus (Shen 1969), ryegrass (Bar-Akiva *et al* 1970) and winter wheat (Verstraeten and Vlassak 1981).

### Experiment 2. The influence of $\text{N}$ form on the yield and $\text{N}$ status of barley plants

#### Shoot fresh and dry weight yields and $\text{N}$ uptake

Fresh and dry weights of shoots were significantly ( $P < 0.05$ ) higher in plants supplied with  $\text{NH}_4^+$  alone or a 1:1 mixture of  $\text{NH}_4^+$  and  $\text{NO}_3^-$  than with  $\text{NO}_3^-$  alone (Table 2). This reflects the greater absorption of  $\text{N}$  from the  $\text{NH}_4^+$ -containing solutions (Table 2). These results confirm the findings of other workers that many plant species absorb  $\text{NH}_4^+$  more rapidly than  $\text{NO}_3^-$ , especially at low root temperatures (Clarkson and Warner 1979). This holds true whether the  $\text{NH}_4^+$  and  $\text{NO}_3^-$  are supplied together or separately (Shen 1969).

TABLE 2

Effect of  $\text{N}$  form on yield, nitrogen content and nitrate reductase activity of winter barley shoots at the 4th leaf stage of growth

|  | Form of nitrogen   |                               |                   |
|--|--------------------|-------------------------------|-------------------|
|  | $\text{NH}_4^+$    | $\text{NH}_4^+/\text{NO}_3^-$ | $\text{NO}_3^-$   |
| Dry weight (g)   | 2.4 <sup>a</sup>   | 2.6 <sup>a</sup>              | 2.1 <sup>b</sup>  |
| Total N (% DM)   | 4.7 <sup>a</sup>   | 4.5 <sup>a</sup>              | 4.1 <sup>b</sup>  |
| $\text{NO}_3^-$ -N (% DM)  | <0.01 <sup>a</sup> | 0.06 <sup>b</sup>             | 0.16 <sup>c</sup> |
| $\text{NRA}_e$ ( $\mu\text{mol NO}_2^- \text{ g FW}^{-1} \text{ h}^{-1}$ )               | 0.07 <sup>a</sup>  | 0.87 <sup>b</sup>             | 1.50 <sup>c</sup> |
| $\text{NRI}_i$ ( $\mu\text{mol NO}_2^- \text{ g FW}^{-1} \text{ h}^{-1}$ )               | 0.10 <sup>a</sup>  | 1.01 <sup>b</sup>             | 1.55 <sup>c</sup> |
| Nitrogen Response Index  | —                  | 1.2 <sup>a</sup>              | 1.1 <sup>b</sup>  |
| Ammonium and nitrate N added to maintain target concentrations during growth period (mg) | 785                | 598/80                        | 615               |

For each parameter, results followed by same letter are not significantly different at the 5% level by least significant difference.

### Nitrate reductase activity

Form of N had a marked influence on NRA (Table 2). There were significant ( $P < 0.01$ ) differences in enzyme activity in plants grown in  $\text{NO}_3^-$  and  $\text{NO}_3^- + \text{NH}_4^+$  treatments. For plants grown in both these treatments, however, NRI approached unity indicating a high N status. These results support the findings of Experiment 1. They also show the superiority of NRI over tissue  $\text{NO}_3^-$  concentration as an indicator of the N status of plants absorbing a large proportion of their N in  $\text{NH}_4^+$  form.

The widely observed inhibitory effect of  $\text{NH}_4^+$  on NRA (Oaks *et al* 1977; Mengel *et al* 1983) was clearly shown in the 100%  $\text{NH}_4^+$ -N treatment where tissue  $\text{NRA}_e$  and  $\text{NRA}_i$  values were so low that NRI was not meaningful. However, it is unlikely that plants growing under field conditions will ever absorb all their N as  $\text{NH}_4^+$ .

## CONCLUSIONS

The results of this study show that NRI can estimate the N status of winter barley plants grown with varied N nutrition under controlled conditions. Since NRI measures the extent to which the rate of N absorption is meeting plant demand for N at the time of sampling, it also reflects the multiplicity of environmental factors—physical, chemical and biological—affecting N availability, uptake and translocation. More work is required to determine if the reported findings also apply to field-grown crops. If they do, the diagnostic use of NRI may have an important role to play in improving the efficiency of fertiliser N usage.

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## REFERENCES

- Allison F E 1966 The fate of nitrogen applied to soils. *Advan Agron* **18** 219–258.
- Bar-Akiva A 1970 Chemical and biochemical measurements on plants as a means of controlling yield and plant performance. *Proc 9th Congr Int Potash Soc* 211–219.
- Bar-Akiva A, Sagiv J, Leshem J 1970 Nitrate reductase activity as an indicator for assessing the nitrogen requirement of grass crops. *J Sci Food Agric* **21** 405–407.
- Clarkson D T, Warner A J 1979 Relationship between root temperature and the transport of ammonium and nitrate ions by Italian and perennial ryegrass (*Lolium multiflorum* and *Lolium perenne*). *Plant Physiol* **64** 557–561.
- Clement C R, Hopper M J, Canaway R J, Jones L H P 1974 A system for measuring the uptake of ions by plants from flowing solutions of controlled composition. *J Exp Bot* **25** 81–99.
- Dowdell R J 1982 Fate of nitrogen applied to agricultural crops with particular reference to denitrification. *Phil Trans Roy Soc London B* **296** 363–373.

- Elsmere J I 1988 Use of fertilisers in England and Wales. *Rothamsted Exp Sta Report for 1987*, Harpenden, Herts.
- Greenwood D J 1982 Modelling of crop response to nitrogen fertiliser. *Phil Trans Roy Soc London B* **296** 351–362.
- Jackson M L 1958 *Soil Chemical Analysis*. Prentice-Hall, Englewood Cliffs, NJ.
- Jenkinson D S 1984 The supply of nitrogen from the soil. In: *The Nitrogen Requirement of Cereals*, MAFF Reference Book 385. HMSO, London, pp 79–92.
- MAFF 1983 *Lime and Fertiliser Recommendations No 1 Arable Crops*. Booklet 2191 Ministry of Agriculture, Fisheries and Food, London.
- Massey D M, Winsor G W 1980 Some responses of tomatoes to nitrogen in recirculating solutions. *Acta Hort* **84** 127–135.
- Mengel K, Robin P, Salsac L 1983 Nitrate reductase activity in shoots and roots of maize seedlings as affected by the form of nitrogen nutrition and the pH of the nutrient solution. *Plant Physiol* **71** 618–622.
- Oaks, A, Aslam M, Boesel I 1977 Ammonium and amino acids as regulators of nitrate reductase in corn roots. *Plant Physiol* **59** 391–394.
- Papastylianou I 1986 Diagnosis of nitrogen deficiency in barley, growing in different rotation systems, by analysis. *Fert Res* **9** 241–250.
- SAC 1985 Fertiliser recommendations. Scottish Agricultural Colleges Publication No 160. SAC, Perth.
- Shen T C 1969 The induction of nitrate reductase and the preferential assimilation of ammonium in germinating rice seedlings. *Plant Physiol* **44** 1650–1655.
- Stanford G 1982 Assessment of soil nitrogen availability. In: *Nitrogen in Agricultural Soils*, ed Stevenson F J. Amer Soc Agron Monograph 22 651–688.
- Sylvester-Bradley R, Barnard P A, Hart P F W 1984 An assessment of nitrate reductase activity as a predictor of nitrogen requirement of winter cereals. In: *The Nitrogen Requirement of Cereals*, MAFF Reference Book 385. HMSO, London, pp 233–238.
- Sym G 1984 Optimisation of the *in vivo* assay conditions for nitrate reductase in barley (*Hordeum vulgare* L cv Igri). *J Sci Food Agric* **35** 725–730.
- Verstraeten L M J, Vlassak K 1981 Nitrogen stress and plant growth in relation to the nitrogen status of plant and soil. *Pedologic* **XXXI** 379–392.

## Protein Quality of Parched Immature Durum Wheat (*Frekeh*)

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### ABSTRACT

*The protein quality of parched immature Durum wheat (frekeh) produced from Deir Alla-2 variety was evaluated. Frekeh from two maturity stages was used: one at the end of the milk stage (F1) and the other at the end of the dough stage (F6). Two animal experiments were carried out in which net protein utilisation (NPU) was determined for the two samples and for mature wheat using Sprague Dawley rats, the first for 12 and the second for 11 days. Additionally, the chemical scores for these cereals were calculated.*

*The results indicated that the protein of F1 was of higher quality than that of F6 or mature wheat; the protein efficiency ratio adjusted values for F1, F6 and wheat were respectively 2.70, 1.93 and 1.45, and the  $NPU_{op}$  values were 53, 46 and 44 respectively. There was agreement between these results and the chemical score values as they were 54, 41 and 42 for F1, F6 and wheat respectively. There was a significantly higher voluntary intake of cereal-based diets in comparison with the casein-based diet ( $P < 0.05$ ).*

*Thus it is shown that frekeh produced from wheat harvested at an early stage of maturity was of higher protein quality than that produced at a late stage of maturity or mature wheat.*

**Key words:** *Frekeh*, immature grains, Durum wheat, cereal products, protein quality, net protein utilisation.

### INTRODUCTION

Cereal products are estimated to cover 50–60% of the daily intake of energy and 40–60% of the daily protein intake in the diet of people in most Middle Eastern

countries (FAO 1984). Wheat is the main cereal produced and consumed in these countries (Patwardhan and Darby 1972; Pellet 1976). Bread and other bakery products are the main wheat products, whereas *burghul* (parboiled immature Durum wheat grains) and *frekeh* (immature Durum wheat grains parched by roasting through ignition without burning of the grains) constitute raw materials for many traditional dishes in Jordan and other Arab countries (Humeid and Umari 1986). Products similar to *frekeh* are also known in other parts of the world; for example *Gruenkern* in Germany is produced from Durum wheat that is specially grown for this purpose (Heimann 1972; Taeufel *et al* 1979).

The annual production of *frekeh* in Middle Eastern countries and North Africa is estimated to be in the range of 200 000–300 000 t (Williams and El Haramien 1985). This production generally follows the traditional methods described by Umari and Humeid (1986). These authors have developed equipment for mechanical parching and have studied the feasibility of mechanical threshing of parched grains at various maturity levels during the dough stage. In a further publication (Humeid and Umari 1986), *frekeh* produced from wheat harvested at six levels of maturity and from mature wheat grains of the same variety was analysed for protein, ash, carbohydrate, fat and soluble sugar content. In addition the time needed for proper cooking and the sensory quality of cooked product was evaluated. It was found that *frekeh* produced at the beginning of the dough stage was richer in nutrients than that produced at the following maturity levels; the earlier the production, the higher were the contents of protein, fat, minerals, crude fibre and reducing sugars. The measured 'standardised' cooking time of the *frekeh* produced at the earliest maturity level (end of milk stage) was a quarter to one-third of the time needed for that produced at the latest maturity level which is still acceptable for *frekeh* making. Corresponding to this, the sensory quality as regards colour and flavour decreased steadily with increasing maturity of the grains from which *frekeh* was produced.

The observed decrease in protein content with increasing maturity level raised the question of how the protein quality of *frekeh* versus that of mature wheat grains varied. In this investigation, the protein efficiency ratio (PER) and the net protein utilisation (NPU) of *frekeh* were determined using rats.

## MATERIALS AND METHODS

Two lots of *frekeh* (parched immature Durum wheat) produced mechanically (Umari and Humeid 1986) at two different maturity stages and mature wheat, all of the local variety Deir Alla-2, were used in the study. The first lot of *frekeh* (F1) was produced at the end of the milk stage and the second (F6) was produced at the end of the dough stage. Protein quality was assessed using net protein utilisation (NPU) and protein efficiency ratio (PER).

The procedure used was that of Miller (1963) with some modifications. The strain of rat suggested by Miller was not available. Instead, 23-day-old male weanling rats of the Sprague-Dawley strain were used. They were fed a stock diet for two weeks and then divided into five groups of five rats each. The differences in mean weight between any two groups did not exceed 2 g. Each rat was individually caged and

maintained at a temperature of  $23 \pm 2^{\circ}\text{C}$ . One group was given a casein diet, the second was fed a protein-free diet, and the other three groups were fed the experimental diets containing F1, F6 and wheat ground to pass through a 1-mm sieve. The composition of diets is shown in Table 1 and the proximate analysis of F1, F6 and wheat is presented in Table 2. The proximate analysis was done according to methods described in AOAC (1980).

Two similar experimental runs were conducted under the same conditions two months apart (July and October 1987). The diets were fed for 12 and 11 days respectively. The animals were weighed and killed by chloroform anaesthesia. The abdomen, thorax and skull of the animals were opened before they were dried in a hot-air oven set at  $105^{\circ}\text{C}$  for 48 h. Body water was calculated and carcass N was then determined by the Kjeldahl method.

The NPU operative was calculated using the Miller equation (Miller 1963):

$$\text{NPU}_{\text{op}} = \frac{[(\text{Carcass N of experimental group} - \text{carcass N of control group fed nitrogen-free diet}) / (\text{Nitrogen intake})] \times 100}$$

TABLE 1  
Composition of the experimental diets fed to different groups of rats (g per 100 g)

| Component                | Diet <sup>a</sup> |     |    |    |    |
|--------------------------|-------------------|-----|----|----|----|
|                          | Cas               | NPD | F1 | F6 | W  |
| Casein                   | 10                | —   | —  | —  | —  |
| Corn starch              | 73                | 83  | —  | —  | —  |
| Corn oil                 | 10                | 10  | 10 | 10 | 10 |
| Salt mix <sup>b</sup>    | 5                 | 5   | 5  | 5  | 5  |
| Vitamin mix <sup>c</sup> | 2                 | 2   | 2  | 2  | 2  |
| Cereal                   | —                 | —   | 83 | 83 | 83 |

<sup>a</sup> Cas, Casein; NPD, non-protein diet; F1, frekeh produced from grains at the end of the milk stage; F6, frekeh produced from grains at the end of the dough stage; W, mature wheat.

<sup>b</sup> Based on Hoppel and Tandler (1975).

<sup>c</sup> In glucose to provide the following  $\text{kg}^{-1}$  diet: vitamin A palmitate 2750  $\mu\text{g}$ ; cholecalciferol 10  $\mu\text{g}$ ;  $\alpha$ -tocopherol 100 mg; ascorbic acid 990 mg; inositol 110 mg; choline chloride 1650 mg; menadione 10 mg; *p*-aminobenzoic acid 110 mg; niacin 99 mg; riboflavin 40 mg; pyridoxine HCl 22 mg; thiamine HCl 20 mg; calcium pantothenate 66 mg; biotin 0.44 mg; folic acid 2 mg; cyanocobalamin 30  $\mu\text{g}$ .

TABLE 2  
Proximate analysis of *frekeh* and wheat used in experimental diets (g per 100 g)

| Cereal | Moisture | Protein | Fat | Ash | Crude fibre | Soluble carbohydrates | Energy <sup>a</sup> (kcal) |
|--------|----------|---------|-----|-----|-------------|-----------------------|----------------------------|
| F1     | 9.7      | 12.3    | 2.5 | 2.0 | 3.8         | 69.7                  | 351                        |
| F6     | 10.2     | 10.3    | 1.6 | 1.6 | 3.3         | 73                    | 348                        |
| W      | 9.5      | 11.2    | 1.9 | 1.7 | 2.3         | 73.4                  | 356                        |

<sup>a</sup> Calculated from proximate analysis values.

The NPU standardised was also calculated using the equation of Miller (1963) and Miller and Payne (1961).

$$\text{NPU standardised} = [(54 \times \text{NPU}_{\text{op}}) / (54 - \text{PE } \%) ] - 8$$

where PE % expresses the energy value of protein in the diet as a proportion of total metabolic energy (Miller and Payne 1959).

The caloric content was calculated using proximate analysis values of the diets and using the figures 4, 9 and 4 kcal g<sup>-1</sup> carbohydrates, fats and protein respectively. Calculation of PER was done according to Campbell (1963). PER values were adjusted by setting the value for casein at 2.5 to obtain PER adjusted values. This is because the standardised conditions described by Chapman (Chapman *et al* 1959) were not followed in this experiment.

Net dietary protein as a percentage of total energy (NDpE %), which is an indicator of both quality and quantity of protein, was calculated according to Miller (1963):

$$\text{NDpE } \% = (\text{NPU}_{\text{op}} / 100) \times [(4 \times 6.25 \text{ N } \%) / \text{kcal per g}]$$

where N % = is the nitrogen percentage.

The chemical scores (CS) for *frekeh* and wheat were calculated according to the formula:  $\text{CS} = A/B$ , where  $A$  = mg limiting amino acid per gram N in the cereal; and  $B$  = mg of the same amino acid per gram N in the FAO reference pattern.

The amino acid values for each cereal product used were those obtained by Humeid *et al* (1989) and assessed against the FAO modified provisional pattern (FAO 1973).

Results of food consumption and weight gain were statistically analysed using Duncan's multiple-range test as given by Little and Hills (1972).

## RESULTS

The food consumption data of rats combined from the two runs of the experiment are shown in Table 3. The food consumption was significantly higher ( $P < 0.01$ ) for rats fed on F1 as compared with casein. There were generally significant differences in the consumption of food between cereal diets and casein ( $P < 0.05$ ). The consumption of *frekeh* (F1, F6) tended to be higher than that of wheat although the differences were not significant ( $P > 0.05$ ).

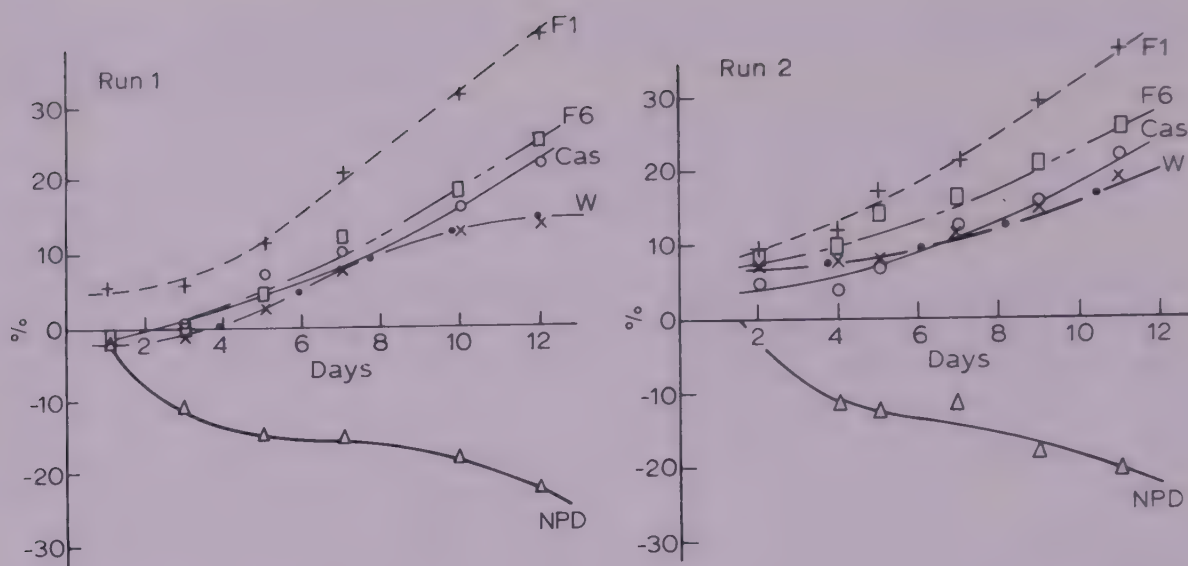
The weight gain data are presented in Fig 1 and Table 3. In both runs of the experiment it was found that the weight gain of the rat groups fed on the F1-based diet was significantly higher than that of all other groups in which the weight gain was similar (except for rats on the protein-free diet where there was a weight loss as expected).

The net protein utilisation (NPU) as well as the weight gain per gram protein consumed (PER) are shown in Table 3. The NPU values were higher for casein than for other diets ( $P < 0.05$ ); the value for F1 was higher than that for F6 or W ( $P < 0.05$ ). PER values, however, were higher in the two runs for F1 than for casein.

TABLE 3  
Total food consumption, net weight gain, NPU and PER of rats fed casein-, frekeh- and wheat-based diets (values are combined from two runs of the experiment)

| Group<br>(no of animals) | Total food<br>consumption<br>(g) | Weight gain<br>(g)       | NPU <sub>op</sub>     | NPU <sub>st</sub> | PER                      | PER <sub>adj</sub> <sup>a</sup> | P cal % | NDpE % |
|--------------------------|----------------------------------|--------------------------|-----------------------|-------------------|--------------------------|---------------------------------|---------|--------|
| Cas (10)                 | 104.6 ± 5.4 <sup>a</sup>         | 17.7 ± 3.5 <sup>ab</sup> | 70 ± 0.8 <sup>a</sup> | 76.5              | 1.90 ± 0.13 <sup>a</sup> | 2.5                             | 9.4     | 6.5    |
| F1 (10)                  | 154.0 ± 5.6 <sup>b</sup>         | 34.8 ± 2.2 <sup>c</sup>  | 53 ± 1.1 <sup>b</sup> | 57.5              | 1.95 ± 0.10 <sup>a</sup> | 2.7                             | 10.5    | 5.6    |
| F6 (10)                  | 147.4 ± 6.1 <sup>b</sup>         | 22.7 ± 1.4 <sup>a</sup>  | 46 ± 0.2 <sup>c</sup> | 47.0              | 1.45 ± 0.08 <sup>b</sup> | 1.93                            | 9.8     | 4.1    |
| W (10)                   | 131.9 ± 5.4 <sup>b</sup>         | 14.8 ± 1.7 <sup>b</sup>  | 44 ± 1.6 <sup>c</sup> | 46.0              | 1.08 ± 0.06 <sup>c</sup> | 1.45                            | 9.8     | 4.2    |

<sup>a</sup> Calculated by setting the value for casein at 2.50 (Campbell 1963).  
Figures are means ± SEM. Values given different letters in the same column are significantly different ( $P < 0.05$ ).



**Fig 1.** Weight gain of rats fed with casein diet, protein-free diet and cereal diets during the experimental period of 12 days (run 1) or 11 days (run 2). F1, *Frekeh* produced from grains at the end of the milk stage; F6, *frekeh* produced from grains at the end of the dough stage; W, mature wheat; Cas, casein; NPD, non-protein diet.

## DISCUSSION

Durum, which is the common wheat in the region, contains an average of 12.7% protein (Krause and Mahan 1984). However, the significance of any dietary protein source is defined by its quality as well as its quantity. It is well established that proteins of cereals, including wheat, are of lower quality than animal protein sources. The NPU and PER are two methods used for evaluating the quality of proteins. The PER and NPU values for wheat proteins were reported to vary in different experiments (Campbell 1963; Takruri 1973; Williams 1985). PER values range from about 0.50 to as high as 1.53 and the NPU values reported in the literature range from 35 to 48.

### Food consumption

As shown in Table 3, the consumption of the F1-based diet was the highest among all of the experimental diets including the casein-based diet. The preference for *frekeh*, especially F1, is probably due to the characteristic flavour and the soft texture of the immature wheat as well as the secondary flavour resulting from parching and light smoking. Smoking is known to improve the palatability of foods (Heimann 1982). It is interesting to note that studies in man on the sensory quality of *frekeh* have shown that *frekeh* produced at the beginning of the dough stage was preferred to that produced at later maturity levels (Humeid and Umari 1986).

### Net protein utilisation and chemical score

The high NPU value of casein, which is an animal protein, is as expected. Casein is classified among the so-called complete proteins. Wheat protein is of lower quality because of poorer amino acid balance due to the low contents of lysine and

**TABLE 4**  
Amino acid scores and chemical score of F1, F6 and wheat

| <i>Essential amino acids</i> | <i>F1</i>       | <i>F6</i>       | <i>W</i>        |
|------------------------------|-----------------|-----------------|-----------------|
| Isoleucine                   | 98              | 92              | 84              |
| Leucine                      | 93              | 96              | 90              |
| Lysine                       | 56 <sup>a</sup> | 41 <sup>b</sup> | 42 <sup>b</sup> |
| Total aromatic amino acids   | 104             | 114             | 110             |
| Total sulphur amino acids    | 119             | 133             | 151             |
| Threonine                    | 54 <sup>b</sup> | 53 <sup>a</sup> | 51 <sup>a</sup> |
| Tryptophan <sup>c</sup>      | —               | —               | —               |
| Valine                       | 88              | 84              | 76              |

<sup>a</sup> Second limiting amino acid.

<sup>b</sup> First limiting amino acid with the corresponding chemical score.

<sup>c</sup> Tryptophan was not measured.

threonine, the first and second limiting amino acids respectively. However, what is interesting in the results of the present study is the relatively high NPU value for F1, which is an immature wheat at the end of the milk stage, as compared with F6 and mature wheat (53, 46 and 44, respectively, taking the average of two runs of the experiment). This result is supported by the chemical scores. The chemical scores for F1, F6 and wheat were 54, 41 and 42, respectively (Table 4). It should be noted here that the chemical scores as well as NPU values were almost the same for wheat and F6 which is produced from grains at late maturity stage.

These results indicate that the amino acid composition varies with maturity stage. The amino acid scores for threonine, the second limiting amino acid in wheat, were almost the same for F1, F6 and mature wheat, whereas only the score for lysine, which is the first limiting amino acid, decreased with maturity. The reason for the change in protein quality could lie in the development programme of the different parts of the grain such as the germ and the aleurone layer with their specific proteins during growth and maturation. It is established that the lysine content of the germ protein is higher than that of gluten which originates in the aleurone layer (Williams 1985).

The NPU value reflects the portion of protein intake which is retained. The NPU operative values are obtained at actual protein levels of the diet and are therefore affected by such levels. For this reason the NPU standardised is used to compare NPU values at maintenance levels of protein (Platt *et al.* 1961). However, since the protein contents in this experiment were similar for all diets, the differences in NPU operative values did not change substantially when NPU was expressed as NPU standardised.

### Protein efficiency ratio

The PER values adjusted for a PER value of 2.50 for casein, were 2.70, 1.93 and 1.45 for F1, F6 and wheat, respectively. The PER for F1 is obviously high. This is in agreement with the high weight gain data given in Fig 1, as the PER is the weight gain per gram of protein consumed. Such a high value indicates the relatively high

protein quality of this wheat product and is in line with the NPU and chemical score results. It is not clear in this experiment why the PER value of the F1-based diet was even higher than that of casein. Variation in PER values, even for the same food, has been reported by Campbell (1963). The main reason for this variation is the short period of the experiment.

Net dietary protein as a percentage of total energy (NDpE %) was used to express both the quality and quantity of dietary proteins. As shown in Table 3, the NDpE % for F1 (5.6 %) was close to that of casein (6.5 %) and substantially higher than that for F6 or wheat (4.1 %, 4.2 %) respectively. Platt *et al* (1961) recommended an NDpE % value of dietary protein mixture of at least 5.9 % for young children and 4.6 % for adults.

The results of protein quality evaluation, namely those of chemical scores, NPU, PER and NDpE %, all suggest that the parched immature wheat is of better protein quality and utilised better in the body as compared with mature wheat. Frekeh's popularity and the fact that it is prepared and cooked in different ways are good reasons for encouraging its production, especially at the early stage of maturity.

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## REFERENCES

- AOAC 1980 *Official Methods of Analysis* (13th edn). Association of Official Analytical Chemists, Washington, DC, pp 132–133, 211, 214 and 858.
- Campbell G A 1963 Methodology for protein evaluation. A critical appraisal of methods for evaluation of protein in foods. American University of Beirut, Lebanon, Faculty of Agricultural Science, Publ No 21.
- Chapman D G, Castello R, Campbell G A 1959 Evaluation of protein in foods. I A method for determination of protein efficiency ratio. *Can J Biol Chem Phys* **37** 679–686.
- FAO 1984. Food Balance Sheets, 1979–81 Average. Food and Agriculture Organization, Rome.
- FAO/WHO Ad Hoc Expert Committee 1973 Energy and Protein Requirements. WHO Tech Rep Ser No 522, Geneva.
- Heimann V 1972 *Grundzuege der Lebensmittelchemie*. Dietrich Steinkopf Verlag, Darmstadt, p 357.
- Heimann W 1982 *Fundamentals of Chemistry*. Ellis Horwood, Chichester, p 311.
- Hoppel E L, Tandler B 1975 Riboflavin and mouse hepatic cell structure and functions: mitochondria oxidative metabolism in severe deficiency state. *J Nutr* **105** 562–570.
- Humeid M, Umari M 1986 A study toward mechanization of frekeh-making in Jordan. II Study of some nutrients and cooking time of frekeh produced at different stages of maturity of wheat heads [in Arabic]. *Dirasat* **13** 73–86.
- Humeid M, Takruri H, Umari M 1989 Amino acid analysis of durum wheat at different maturity levels of the grains suitable for frekeh-making. *Dirasat* in press.
- Krause M V, Mahan L K 1984 *Nutrition and Diet Therapy* (7th edn). W B Saunders, Philadelphia, PA, p 901.

- Little T, Hills J 1972 *Statistical Methods in Agricultural Research*. Agricultural Extension, University of California, Davis, CA, pp 50–51.
- Miller D S 1963 A procedure for determination of NPU using rats body N technique. In: *Evaluation of Protein Quality*, Nat Acad Sci Nat Res Council, Publ No 1100, Washington, DC, pp 34–36.
- Miller D S, Payne P R 1959 A ballistic bomb calorimeter. *Br J Nutr* **13** 501–508.
- Miller D S, Payne P R 1961 Problems in the prediction of protein value of diets. The influence of protein concentration. *Br J Nutr* **15** 11–19.
- Patwardhan V N, Darby V G 1972 *The State of Nutrition in the Arab Middle East*. Vanderbilt University Press, Nashville, Tenn.
- Pellet P L 1976 Nutritional problems of the Arab world. *Ecol Food Nutr* **5** 205–215.
- Platt B S, Miller D S, Payne P R 1961 Protein values of human food. In: *Recent Advances in Human Nutrition*, ed J F Brock. J & A Churchill, London, pp 351–374.
- Taeufel A, Tunger L, Zobel M (Eds) 1979 *Lebensmittel Lexikon*. VEB-Verlag, Leipzig, p 355.
- Takruri H 1973 The development of high-protein biscuit for school-feeding programs. MSc Thesis, American University of Beirut, pp 39–40.
- Umari M, Humeid M 1986 A study toward mechanization of frekeh-making in Jordan. I Construction of mechanical parching and threshing devices [in Arabic], *Dirasat* **13** 32–51.
- Williams P C, El-Haramien F G 1985 Frekeh-making in Syria. *ICARDA, Aleppo, RACHIS* **4** 25–27.
- Williams R 1985 *Nutrition and Diet Therapy* (6th edn). C V Mosby, St Louis, MO, p. A40.



## Comparison of the Polyphenol Profiles of Apple Fruit Cultivars by Correspondence Analysis

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### ABSTRACT

*Concentrations of the major flavonoids and phenolic acids in the peel and cortex of fruit of eight commercial apple cultivars were determined by HPLC. The multivariate statistical technique of correspondence analysis was applied to the polyphenol profiles to describe distinctive groups of cultivars and of polyphenols, and their joint correspondences.*

*Cultural and growing conditions had a limited effect on the polyphenol profiles of the cortex and peel. Chlorogenic acid was the principal polyphenol in the cortex with the lowest levels being in Red Delicious and the highest in Jerseymac, which were compensated by changes in phlorizin. Cortland had low levels of chlorogenic acid and Gravenstein had high levels, but these were offset by the levels of catechins.*

*The quercetin, rhamnoside, was the principal phenolic compound for the peel data with low levels in Red Delicious, Cortland, Spartan and Jerseymac and high levels in Golden Delicious, Gravenstein and Northern Spy. Levels of chlorogenic acid, offset by levels of phlorizin and catechins, distinguished between Red Delicious and Cortland. Rutin was important in distinguishing between Jerseymac and Spartan.*

**Key words:** Apple, flavonoids, phenolic acids, correspondence analysis.

## INTRODUCTION

Polyphenol levels, particularly of flavonoids, show great promise as taxonomic markers for biochemical systematic analysis of plants (Denford 1984). In most systematic studies of flavonoids, only the presence or absence of a particular character has been determined (Harborne 1975). The specificity of quantitative methods for systematic studies of plants was shown by the characteristic pattern of leaf constituents among species (Challice and Williams 1968). The analysis of intraspecific variation will likely depend on quantitative analysis; a good illustration is given by the HPLC of flavonol glycosides in sepals of poinsettia (Stewart *et al* 1980). The phenolics of *Malus* species fruits have been qualitatively surveyed (Teuber Wuenscher and Herrmann 1978), and the HPLC methodology for their resolution and identification is well established (Lea 1982; Dick *et al* 1987).

The present paper describes the quantities of the major phenolic acids and flavonoids in the peel and cortex of the fruit of eight apple cultivars grown commercially in Eastern Canada. The statistical procedure of correspondence analysis (Greenacre 1984) is used to examine associations among cultivars with the principal distinguishing characteristics of the polyphenolic profiles they contain. Correspondence analysis may be useful to taxonomists for characterising apple cultivars based on polyphenol profiles in the peel and cortex of the fruit.

## MATERIALS AND METHODS

Commercial apple orchards in the Annapolis Valley of Nova Scotia, Canada, comprise many combinations of different rootstocks, managements, fertilities and soils throughout the fruit-producing area. For each of the following cultivars, from each of five orchards, 25-fruit samples (preclimacteric to climacteric) were taken: Gravenstein, McIntosh, Cortland, Spartan, Golden Delicious and Northern Spy. Similarly, mature samples of the cultivar Jerseymac were taken from four orchards.

### Measurements

Ethylene evolution after harvest was determined for all the samples except for those of Northern Spy (see Table 1). Ten apples from each orchard were weighed and placed in 4-litre air-tight polyethylene jars with an ethylene-free airflow of 1 litre  $\text{h}^{-1}$  passed through the jars at 20°C. After 24 h from harvest, a 1-ml sample of headspace effluent was taken and analysed for ethylene using a Carle gas 211 chromatograph (He carrier flow 30 ml  $\text{min}^{-1}$ ), equipped with a 2.5-m alumina column maintained at 70°C, and an FID detector. On a further subsample of ten apples, firmness was determined with a Ballauf penetrometer and titratable acidity was determined with sodium hydroxide to a pH endpoint of 8.1, as previously described (Lidster *et al* 1981).

The remaining five apples from each sample were peeled and opposite sectors were separated for polyphenol analysis. Composite samples of 5.0 g of well-scraped peel and 10.0 g of cortical tissue sectors (less leathery pericarp) for each replication were extracted with 100 ml methanol using a Brinkman Polytron homogeniser, the

homogenate was filtered, the solid residue was extracted with a further 100 ml methanol, and the combined filtrate was evaporated to dryness *in vacuo* at 30°C. The residue was prepared for HPLC analysis by dissolution in methanol to a volume of 25.0 ml. Subsamples of 10.0 µl of the prepared samples were injected into a Varian 5000 liquid chromatograph fitted with a Waters NovaPack C<sub>18</sub> reversed-phase cartridge. The solvent programme consisted of solvent A: tetrahydrofuran (THF, HPLC grade) and solvent B: 1 g litre<sup>-1</sup> trifluoroacetic acid (TFA, HPLC grade) in deionised double-distilled water. The solvents used were programmed as a concave gradient in steps of (solvent A/solvent B v/v): 20/80, 25/75, 35/65, 60/40, 75/25, 100/0, adjusted at time intervals of 0, 5, 10, 15, 17 and 20 min, respectively. Solvent flow was maintained at 1.5 ml min<sup>-1</sup> with the effluent monitored at 270 nm using a Varian UV50 variable wavelength detector connected to a Waters 840 Data Station. The chromatogram peaks were identified by comparing retention times with previously isolated authentic compounds from apples (Dick *et al* 1987). Integrated peaks were calculated by comparison with standard solutions of the authentic compounds.

### Statistical methods

The data formed a matrix with the samples, grouped by cultivar, as rows and the phenolic concentrations as columns. The structure of a data matrix of non-negative values is described by correspondence analysis (Greenacre 1984) by transforming the matrix into a series of single dimensional arrays that portray the row and column characteristics simultaneously. The major characteristic of the matrix structure is identified in the first dimension and portrayed on the first axis, the next most important in the second dimension and so on. For the matrix of samples by phenolic profiles, the samples that had similar profiles would be plotted close together (in a certain sense) in each dimensional display, and those dissimilar would be plotted further apart. Likewise, phenolic compounds plotted close together would have a consistent relationship to each other over the various apple samples.

The simultaneous display of the cultivar samples and the phenolic compounds is justified by the transition formulae, whereby one is expressed in terms of the other. The joint display indicates groups of samples and groups of compounds which lead to an investigation of associations. The geometric interpretation of correspondence analysis is given in Greenacre and Hastie (1987). A useful discussion of correspondence analysis and its variants is given in Digby and Kempton (1987).

### Correspondence analysis algorithm

Values of the  $n$  rows by  $m$  columns data matrix are divided by the grand total; this matrix is centred symmetrically by subtracting the product of row and column totals. The resultant matrix is then pre- and post-multiplied by diagonal matrices  $R^{-1/2}$  and  $C^{-1/2}$  formed from the square roots of the row and column totals, respectively, to obtain the  $(n \times m)$  matrix  $Y$ . The next step is to compute the singular value decomposition (SVD) of  $Y$  to obtain (where pre- and post-subscripts denote matrix dimensions)

$${}_nY_m = {}_nU_m S_m V'_m$$

where  ${}_nU_m$  and  ${}_mV_m$  are the matrices of left and right singular vectors, respectively, and  ${}_mS_m$  is a diagonal matrix of singular values,

$${}_mS_m = \text{diag}(\alpha_1, \alpha_2, \dots, \alpha_{m-1}, 0) \text{ with } \alpha_1 \geq \alpha_2 \geq \dots \alpha_{m-1} \geq 0$$

The rows of the matrices  ${}_nF_m = {}_nR_n^{-1/2} {}_nU_m {}_mS_m$  and  ${}_mG_m = {}_mC_m^{-1/2} {}_mV_m {}_mS_m$  provide coordinates for plotting the  $n$  samples (rows) and the  $m$  compounds (columns). The correspondence analysis in  $p$  dimensions uses the submatrices  ${}_nF_p$  and  ${}_mG_p$  consisting of the first  $p$  columns of the respective matrices. The mean coordinates, in two or three dimensions, for cultivars and compounds are given in Tables 5 and 6, respectively. In correspondence analysis the quantity  $I = \alpha_1^2 + \alpha_2^2 + \dots + \alpha_{m-1}^2$ , the sum of the squared singular values, is known as the inertia of the data matrix, and the proportion of the inertia explained by the  $p$ th axis is the  $p$ th squared singular value ( $\alpha_p^2$ ) divided by  $I$ .

The inertia of the data matrix can be decomposed in several ways, including by individual matrix element, by cases and by compounds. This decomposition is analogous to the partitioning of the  $\chi^2$  for contingency tables. The quantity *mass* for samples and compounds indicates the weight given to each in the analysis. The relative contributions to individual axes (*correlation*) is calculated for cases and compounds. The sum of the correlations over the  $p$  axes equals *quality* (in Tables 5 and 6), and gives the adequacy of the representation. When the inertia of a sample can be described well by a few axes, the quality of the representation will be close to 1000 permills (thousandths). Contributions to an axis add to 1, or 1000 permills, and large values that are associated with points distant from the origin principally determine the position of the axes.

### Interpreting correspondence analyses

Underhill and Peisach (1985) give three principles for interpreting correspondence analysis plots, adapted for the present study, when the quality of representation is high.

- Samples that are represented by being close together tend to be similar in their profiles of polyphenol concentrations. Likewise, polyphenols that are plotted close together tend to be similar among samples.
- The distance between a sample point and a polyphenol point has no interpretation whatsoever. The joint interpretation of sample points and polyphenol points is through the transition formulae. Expressed graphically, they state that sample points are attracted away from the origin in the direction of the polyphenols for which they have high scores.
- Points plotted near the origin have average values for all the polyphenols or alternatively are poorly represented. This can be decided by checking whether the quality of the representation is good or bad, respectively.

### Application of correspondence analysis

The data matrix was transformed prior to the correspondence analysis to give equal weight (mass in Tables 5 and 6) to each polyphenol; the values in each column were divided by the column mean. This standardisation reduced the undesirable effect of

the highly concentrated polyphenols overwhelming those at low concentrations; deviations from the average level of individual compounds were emphasised, thereby identifying distinctive characteristics of individual cultivar profiles. As an aid to the formal statistical analysis, profiles for each sample were calculated by expressing the standardised values as a percentage of the mean of the standardised values in each row (Tables 3 and 4). For example, in Table 3,

$$126\% = 100\% \times (426/238)/(426/238 + 16/17 + 300/196)/3$$

Correspondence analysis was applied to both the cortex and the peel data sets. For presentation in this paper, the individual points were displayed on the graphs of the first 2 (cortex) or 3 (peel) axes. Also, to reduce the size of the tables, only the cultivar mean values were presented; mass, inertia and contributions for the samples were summed, but quality, correlations and coordinates were averaged.

To pick out the distinctive characteristics of the data matrix, the samples and polyphenols that made large contributions to the inertia of an axis were identified. The magnitude and the sign of the coordinates were noted; samples or polyphenols with similar extreme coordinates were formed into groups. Coordinates near zero indicated the sample or polyphenol did not play an important role in determining the axis, and would be poorly represented on that axis if the quality of representation was poor.

The calculations for this study were performed in Genstat 4 (1983), using the singular value decomposition directive within a macro written by the senior author.

RESULTS

Maturity of the cultivars at harvest, as determined by the headspace ethylene, is given in Table 1. Cultivar Jersey mac was mature, Golden Delicious was preclimacteric with a value less than  $1.0 \mu\text{l C}_2\text{H}_4 \text{ kg}^{-1} \text{ h}^{-1}$ , and the remaining cultivars were early climacteric. Determinations were not taken on Northern Spy. Fruit firmness ranged from 69.1 N for Gravenstein to 87.6 N for Red Delicious.

TABLE 1  
Internal ethylene levels, fruit firmness and titratable acids content of apple cultivars at harvest

| Cultivar         | Headspace $\text{C}_2\text{H}_4$<br>( $\mu\text{l kg}^{-1} \text{ h}^{-1}$ ) | Firmness<br>(N) | Titratable acids<br>(mg malic 100 ml <sup>-1</sup> juice) |
|------------------|--|-----------------|---|
| Jersey mac       | 125  | 74.4            | 648   |
| Cortland         | 5.2  | 73.5            | 738   |
| Gravenstein      | 4.4  | 69.1            | 681   |
| Spartan          | 3.1  | 76.8            | 742   |
| McIntosh         | 1.1  | 71.8            | 946   |
| Red Delicious    | 1.0  | 87.6            | 409   |
| Golden Delicious | 0.3  | 77.8            | 616   |
| Northern Spy     | —  | —               | —   |

Titrateable acidity was least for Red Delicious (409 mg malic acid 100 ml<sup>-1</sup> juice) and greatest for McIntosh (946 mg malic acid 100 ml<sup>-1</sup> juice).

### Levels of flavonoids and phenolic acids in fruit

The flavonoids and phenolic acids levels in the eight apple cultivars of this study were resolved by RP-HPLC (Table 2). The concave-shaped solvent program effectively compressed the later eluting components of the chromatogram. Baseline separation was achieved except for the catechins being unresolved (Dick *et al* 1987). The chlorogenic acid isomer identified was 5-(3,4-dihydroxycinnamoyl)-L-quinic acid.

Levels of the determined flavonoids and phenolic acids in the eight apple cultivars are given in Table 3 for the cortex and Table 4 for the peel. High concentrations of chlorogenic acid and catechins were present in the cortex, but low concentrations of phlorizin (an order of magnitude less), and no detectable levels of quercetin. Chlorogenic acid levels ranged from 89 µg g<sup>-1</sup> in the cultivar Cortland to 426 µg g<sup>-1</sup> in Jersey mac. Cortland and Golden Delicious had the lowest concentration of catechins (129 and 130 µg g<sup>-1</sup>), and Jersey mac, with 300 µg g<sup>-1</sup>, had the highest. Quercetin glycosides were not detected in the cortex.

In the peel, high concentrations of the catechins, quercetin arabinoside and the quercetin glucoside + galactoside mixture were observed (Table 4). Ranges for the catechins were from 455 µg g<sup>-1</sup> (Gravenstein) to 1431 µg g<sup>-1</sup> (Red Delicious), for quercetin arabinoside 508 µg g<sup>-1</sup> (Gravenstein) to 801 µg g<sup>-1</sup> (Northern Spy), and quercetin glucoside + galactoside levels ranged from 554 µg g<sup>-1</sup> (Jersey mac) to 924 µg g<sup>-1</sup> (Northern Spy). Chlorogenic acid had the lowest concentration of the

**TABLE 2**  
Retention times of the flavonoids and phenolic acids of apple cultivars on RP-HPLC<sup>a</sup>

| Polyphenol                    | Retention time (min, n = 16) |      |
|-------------------------------|------------------------------|------|
|                               | Mean                         | SD   |
| Chlorogenic acid <sup>b</sup> | 4.61                         | 0.11 |
| Catechins                     | 5.51                         | 0.14 |
| Rutin                         | 7.64                         | 0.13 |
| Quercetin:                    |                              |      |
| Glucoside + galactoside       | 9.43                         | 0.21 |
| Xyloside                      | 11.54                        | 0.52 |
| Rhamnoside                    | 14.50                        | 0.62 |
| Arabinoside                   | 15.36                        | 0.15 |
| Phlorizin                     | 15.89                        | 0.09 |
| Quercetin                     | 17.55                        | 0.22 |

<sup>a</sup> See 'Materials and methods' for chromatography conditions.

<sup>b</sup> Chlorogenic acid isomer identified was 5-(3,4-dihydroxycinnamoyl)-L-quinic acid in the IUPAC 1976 nomenclature (Biochem J 153 23-31).

TABLE 3

Concentration of phenolic compounds in the fruit cortex of eight apple cultivars collected from different orchards in the Annapolis Valley of Nova Scotia. The values were standardised to the column mean prior to calculating the percentage profile for each cultivar

| Cultivar             | Samples | Chlorogenic acid | Phlorizin | Catechins | Mean |
|----------------------|---------|------------------|-----------|-----------|------|
| $\mu\text{g g}^{-1}$ |         |                  |           |           |      |
| Jerseymac            | 4       | 426              | 16        | 300       | 247  |
|                      |         | 126 %            | 66 %      | 108 %     |      |
| Gravenstein          | 5       | 292              | 14        | 143       | 150  |
|                      |         | 133 %            | 88 %      | 79 %      |      |
| Northern Spy         | 5       | 319              | 18        | 275       | 204  |
|                      |         | 106 %            | 83 %      | 111 %     |      |
| McIntosh             | 5       | 268              | 16        | 238       | 174  |
|                      |         | 104 %            | 85 %      | 111 %     |      |
| Golden Delicious     | 5       | 172              | 17        | 130       | 106  |
|                      |         | 91 %             | 125 %     | 84 %      |      |
| Spartan              | 5       | 242              | 21        | 167       | 143  |
|                      |         | 99 %             | 118 %     | 83 %      |      |
| Cortland             | 5       | 89               | 11        | 129       | 76   |
|                      |         | 66 %             | 115 %     | 119 %     |      |
| Red Delicious        | 5       | 97               | 25        | 186       | 103  |
|                      |         | 44 %             | 155 %     | 101 %     |      |
| Mean                 |         | 238              | 17        | 196       | 150  |

determined polyphenols but ranged from 20  $\mu\text{g g}^{-1}$  (Golden Delicious) to 149  $\mu\text{g g}^{-1}$  (Cortland).

Phenolic profiles in cortex

In the correspondence analysis, the first axis represented 75.0% of the total inertia (Fig 1 and Table 5). Jerseymac, Gravenstein and Red Delicious were the principal cultivars, and chlorogenic acid and phlorizin were the principal compounds for determining the axis, as indicated by their high contributions to the inertia of axis 1. High levels of chlorogenic acid were associated with cultivars Jerseymac and Gravenstein in contrast to high levels of phlorizin in Red Delicious (Table 3). Other cultivars had intermediate levels of chlorogenic acid and phlorizin with the mixture changing with the distance from the extremes. On the second axis (25.0% of the total inertia) Gravenstein, Spartan and Golden Delicious were associated with low levels of catechins in contrast to higher levels in Cortland. The associations between cultivar groups and the corresponding phenolic groups may be corroborated from the percentage profiles (Table 3).

Phenolic profiles in peel

For the compounds in peel (Table 6, Figs 2 and 3), three axes explained 85.3% of the total inertia with 48.6, 24.3 and 12.4% by the respective axes. The samples of cultivars, grown under different conditions, formed natural groups on the principal three axes, except for one sample of Gravenstein and one of Golden Delicious

**TABLE 4**  
Concentration ( $\mu\text{g g}^{-1}$ ) of the phenolic compounds in the fruit peel of eight apple cultivars collected from different orchards in the Annapolis Valley of Nova Scotia. The values were divided by the column mean prior to calculating the cultivar profile as percentages

| Cultivar         | Samples | Chlorogenic acid |           |            | Quercetin glycosides |          |                         |             | Mean |
|------------------|---------|------------------|-----------|------------|----------------------|----------|-------------------------|-------------|------|
|                  |         | Phlorizin        | Catechins | Rhamnoside | Rutin                | Xyloside | Glucoside + galactoside | Arabinoside |      |
| Red Delicious    | 5       | 91               | 1431      | 104        | 117                  | 210      | 604                     | 546         | 429  |
|                  |         | 86%              | 165%      | 35%        | 83%                  | 94%      | 78%                     | 82%         |      |
| Spartan          | 5       | 126              | 677       | 71         | 57                   | 244      | 783                     | 662         | 353  |
|                  |         | 140%             | 99%       | 28%        | 47%                  | 129%     | 119%                    | 117%        |      |
| Cortland         | 5       | 149              | 549       | 80         | 159                  | 192      | 735                     | 589         | 322  |
|                  |         | 166%             | 80%       | 32%        | 133%                 | 102%     | 112%                    | 104%        |      |
| Jerseymac        | 4       | 121              | 929       | 128        | 185                  | 119      | 554                     | 556         | 342  |
|                  |         | 134%             | 135%      | 50%        | 153%                 | 62%      | 84%                     | 97%         |      |
| McIntosh         | 5       | 123              | 554       | 361        | 117                  | 254      | 696                     | 777         | 379  |
|                  |         | 119%             | 70%       | 124%       | 85%                  | 117%     | 92%                     | 119%        |      |
| Golden Delicious | 4       | 20               | 590       | 369        | 110                  | 195      | 869                     | 539         | 363  |
|                  |         | 22%              | 86%       | 146%       | 91%                  | 103%     | 132%                    | 95%         |      |
| Gravenstein      | 4       | 61               | 455       | 440        | 139                  | 145      | 594                     | 508         | 323  |
|                  |         | 66%              | 65%       | 169%       | 112%                 | 75%      | 88%                     | 87%         |      |
| Northern Spy     | 5       | 97               | 800       | 661        | 169                  | 297      | 924                     | 801         | 480  |
|                  |         | 77%              | 84%       | 187%       | 101%                 | 113%     | 101%                    | 101%        |      |
| Mean             |         | 99               | 748       | 277        | 132                  | 207      | 720                     | 622         | 374  |

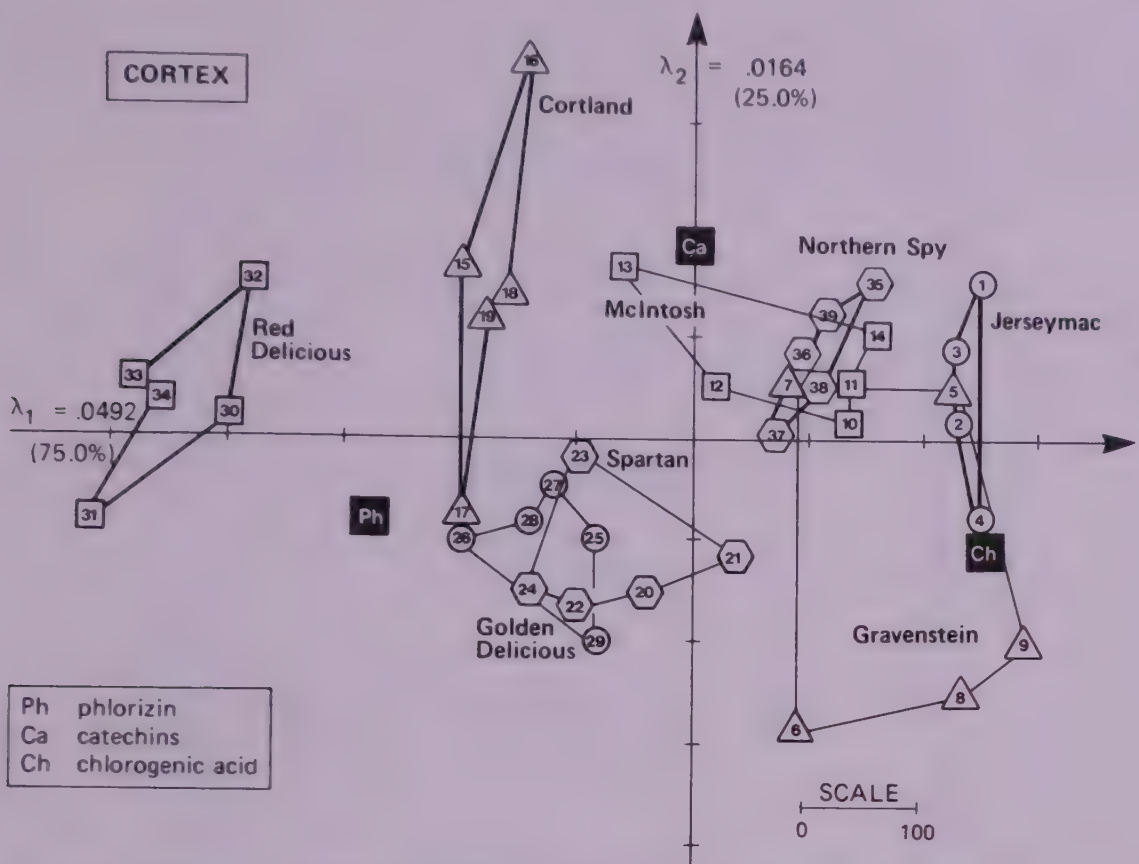


Fig. 1. Correspondence analysis of polyphenol profiles in the fruit cortex of eight apple cultivars, axis 2 versus 1.

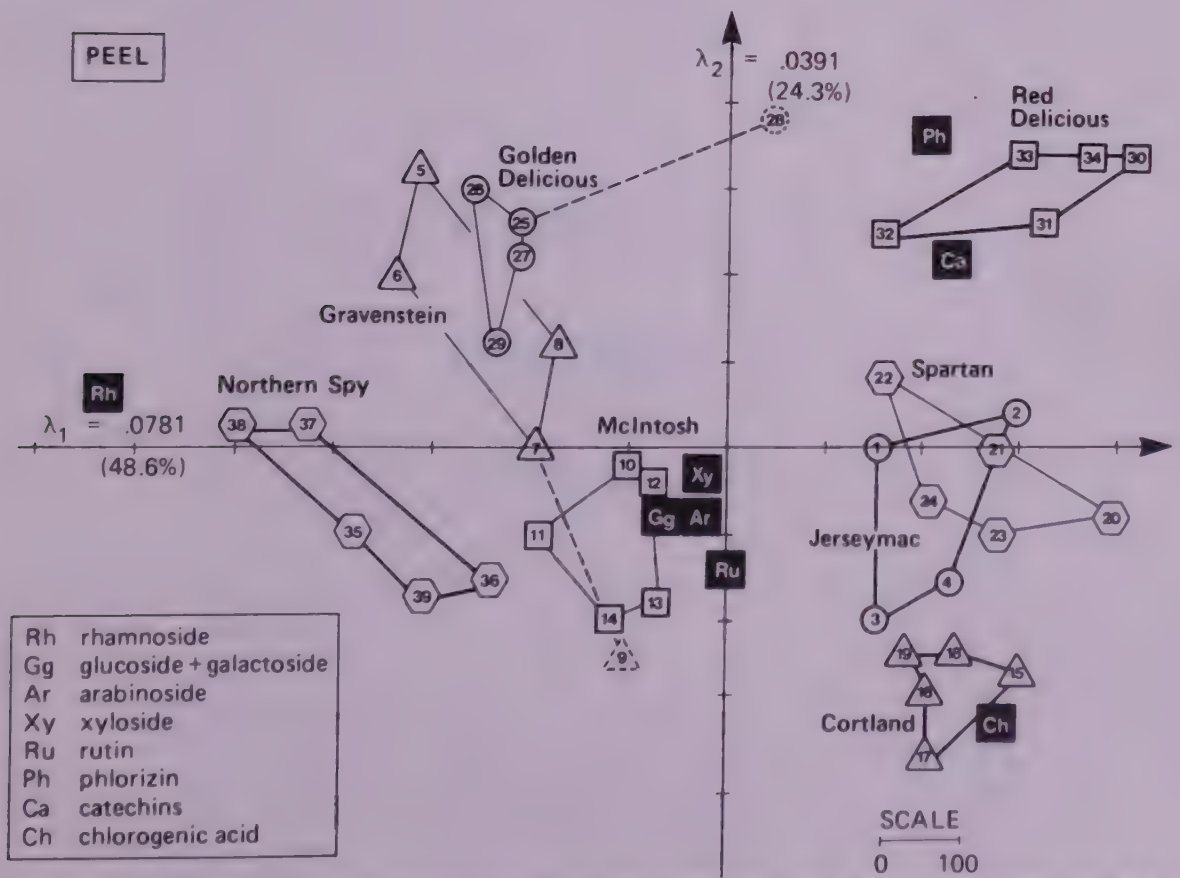


Fig. 2. Correspondence analysis of polyphenol profiles in the fruit peel of eight apple cultivars, axis 2 versus 1.

TABLE 5  
Decomposition of inertia in the correspondence analysis of the cortex data for the first two principal axes. All quantities are expressed as permills (thousandths)

| Cultivar         | Total |              | Axis 1  |            |           | Axis 2       |            |                        |
|------------------|-------|--------------|---------|------------|-----------|--------------|------------|------------------------|
|                  | Mass  | Mean quality | Inertia | Coordinate | Mean corr | Contribution | Coordinate | Mean corr Contribution |
| Jerseymac        | 148   | 1000         | 163     | 248        | 882       | 186          | 45         | 118 92                 |
| Gravenstein      | 129   | 1000         | 160     | 200        | 608       | 111          | -148       | 392 305                |
| Northern Spy     | 172   | 1000         | 56      | 113        | 690       | 44           | 81         | 310 94                 |
| McIntosh         | 132   | 1000         | 48      | 78         | 571       | 37           | 77         | 429 77                 |
| Golden Delicious | 106   | 1000         | 47      | -126       | 589       | 37           | -103       | 411 82                 |
| Spartan          | 128   | 1000         | 51      | -74        | 383       | 25           | -116       | 617 133                |
| Cortland         | 72    | 1000         | 76      | -173       | 568       | 44           | 145        | 432 174                |
| Red Delicious    | 113   | 1000         | 396     | -453       | 966       | 514          | 21         | 34 44                  |
| Polyphenols      |       |              |         |            |           |              |            |                        |
| Chlorogenic acid | 333   | 1000         | 409     | 267        | 888       | 483          | -95        | 112 183                |
| Phlorizin        | 333   | 1000         | 425     | -276       | 912       | 516          | -86        | 88 151                 |
| Catechin         | 333   | 1000         | 167     | 9          | 2         | 1            | 181        | 998 666                |

The total inertia is 0.0656 and the first two principal inertias are 0.0492 (75%) and 0.0164 (25%), respectively.

TABLE 6  
Decomposition of inertia in the correspondence analysis of the peel data for the first three axes. All quantities are expressed as permills (thousandths)

| Cultivar           | Total |              |         | Axis 1     |           |              | Axis 2     |           |              | Axis 3     |           |              |
|--------------------|-------|--------------|---------|------------|-----------|--------------|------------|-----------|--------------|------------|-----------|--------------|
|                    | Mass  | Mean quality | Inertia | Coordinate | Mean corr | Contribution | Coordinate | Mean corr | Contribution | Coordinate | Mean corr | Contribution |
| Red Delicious      | 144   | 909          | 204     | 323        | 467       | 207          | 302        | 420       | 343          | 40         | 22        | 44           |
| Spartan            | 122   | 895          | 133     | 270        | 454       | 124          | -40        | 28        | 16           | -251       | 413       | 433          |
| Cortland           | 122   | 913          | 121     | 232        | 377       | 87           | -278       | 491       | 248          | 27         | 46        | 49           |
| Jerseymac          | 99    | 886          | 93      | 219        | 337       | 65           | -90        | 130       | 47           | 243        | 420       | 319          |
| McIntosh           | 142   | 624          | 52      | -113       | 240       | 28           | -119       | 299       | 71           | -70        | 86        | 38           |
| Golden Delicious   | 98    | 808          | 95      | -241       | 393       | 74           | 207        | 375       | 121          | -70        | 40        | 47           |
| Gravenstein        | 102   | 680          | 100     | -253       | 414       | 89           | 150        | 191       | 89           | 70         | 75        | 42           |
| Northern Spy       | 171   | 878          | 202     | -375       | 758       | 326          | -86        | 101       | 65           | 43         | 20        | 27           |
| <i>Polyphenols</i> |       |              |         |            |           |              |            |           |              |            |           |              |
| Chlorogenic acid   | 125   | 864          | 166     | 295        | 408       | 139          | -312       | 457       | 312          | 9          | 0         | 0            |
| Phlorizin          | 125   | 857          | 158     | 206        | 208       | 68           | 359        | 631       | 412          | -60        | 18        | 22           |
| Catechin           | 125   | 767          | 132     | 248        | 363       | 98           | 223        | 295       | 160          | 136        | 109       | 116          |
| Rutin              | 125   | 779          | 91      | -2         | 0         | 0            | -133       | 151       | 57           | 272        | 628       | 464          |
| <i>Glucoside +</i> |       |              |         |            |           |              |            |           |              |            |           |              |
| galactoside        | 125   | 421          | 52      | -42        | 27        | 3            | -74        | 81        | 17           | -144       | 313       | 131          |
| Xyloside           | 125   | 786          | 34      | -32        | 23        | 2            | -47        | 50        | 7            | -176       | 713       | 195          |
| Arabinoside        | 125   | 665          | 18      | -16        | 11        | 0            | -83        | 295       | 22           | -91        | 359       | 52           |
| Rhamnoside         | 125   | 976          | 349     | -657       | 959       | 690          | 66         | 10        | 14           | 55         | 7         | 19           |

The total inertia is 0.1218 and the first three principal inertias are 0.0781 (48.6%), 0.0391 (24.3%) and 0.0199 (12.4%), respectively.

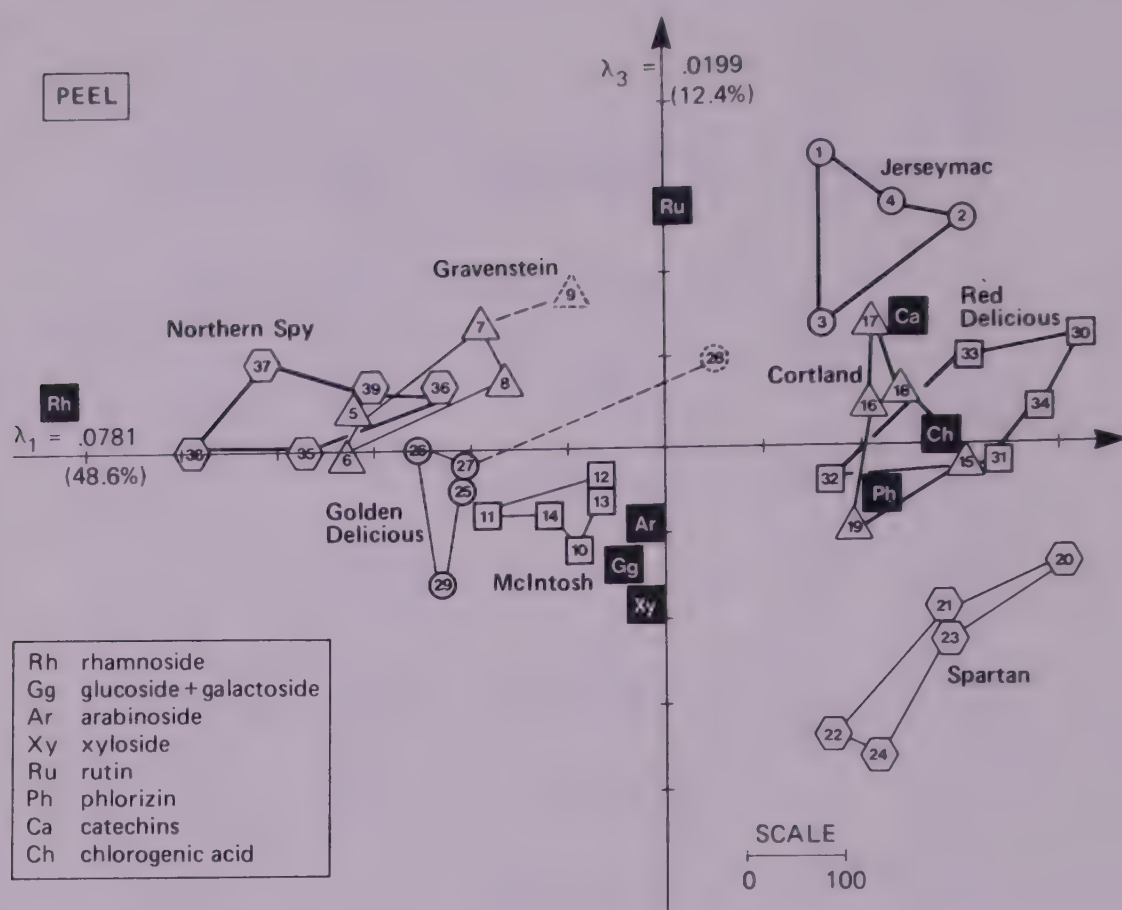


Fig. 3. Correspondence analysis of polyphenol profiles in the fruit peel of eight apple cultivars. axis 3 versus 1.

(samples 9 and 28, respectively, in Figs 2 and 3) which were deleted from the computations for determining the axes but were plotted as supplementary points. Quercetin rhamnoside was the principal compound determining the first axis and was complemented by combinations of chlorogenic acid, catechins and phlorizin. Cultivars Red Delicious, Spartan, Cortland and Jersey Mac had low proportions of quercetin rhamnoside (and high levels in some of the complementary compounds) in contrast to cultivars Northern Spy, Gravenstein, Golden Delicious and McIntosh with high proportions.

The second axis was determined principally by contrasts among the complementary group of compounds on the first axis: phlorizin and catechins, versus chlorogenic acid and corresponding differences among Red Delicious, Cortland, Golden Delicious and to a lesser degree Gravenstein. Red Delicious had high levels of both phlorizin and catechins. Gravenstein and Golden Delicious had high levels of phlorizin but low levels of chlorogenic acid, while Cortland (also Jersey Mac and Spartan) had high levels of chlorogenic acid. Cultivars Spartan and Jersey Mac (associated with rutin, quercetin xyloside, quercetin glucoside + galactoside and catechins) principally determined the third axis (Table 6 and Fig 3). Spartan had a low level of rutin with complementary higher levels of quercetin

xyloside and quercetin glucoside + galactoside. Conversely, the Jersey mac profile had a high level of rutin and catechins but low levels of the other two components.

## DISCUSSION

The mean level of phenolic compounds in the apple cortex differed greatly among the cultivars in this study, from a low of  $76 \mu\text{g g}^{-1}$  in Cortland to a high of  $247 \mu\text{g g}^{-1}$  in Jersey mac; mean levels varied less in the peel. The distinctiveness of individual cultivars will depend, in part, on the quantitative relationships among the phenolic compounds. Correspondence analysis provided a simple means to identify in order of importance the distinctive components in the profiles among the cultivars. Chlorogenic acid, phlorizin and catechins were present in both cortex and skin but quercetin glycosides were detectable only in the peel. For the cortex tissue, Red Delicious was the most distinctive cultivar, with a low level of chlorogenic acid and a high level of phlorizin. Gravenstein was the next most distinctive with a high level of chlorogenic and low levels of phlorizin and catechins. Jersey mac was next, with a high level of chlorogenic acid but a low level of phlorizin. Cortland had high levels of phlorizin and catechins but a low level of chlorogenic acid. Cultivars McIntosh, Spartan, Northern Spy and Golden Delicious had the least distinctive polyphenolic profile features in the cortex.

Samples of the same cultivar tended to form separate groups in the peel except for one Gravenstein and one Golden Delicious sample which were not used in the analysis of the peel (apart from being plotted). On the first axis (48.6%), Red Delicious, Jersey mac, Spartan and Cortland formed one group corresponding to high levels in at least one of phlorizin, catechin and chlorogenic acid, but all had low levels of the quercetin rhamnoside. Conversely, cultivars Northern Spy, Gravenstein and Golden Delicious were the first group due to a higher contribution of the quercetin rhamnoside, and therefore lower levels of the first polyphenol group. The cultivar McIntosh was intermediate. On the second axis, cultivar Red Delicious (and, to a lesser extent, Golden Delicious and Gravenstein) were associated with high levels of phlorizin and catechin in contrast to Cortland which had a high level of chlorogenic acid. Although Spartan and Jersey mac were grouped together in the first two axes, they were distinct in the third axis due to Jersey mac having high levels of rutin (and, to a lesser extent, catechin) in contrast to Spartan having higher levels of the quercetin xyloside and quercetin glucoside + galactoside. Quercetin arabinoside contributed little to the total inertia nor to the individual axes, indicating similar proportions among cultivars.

Samples represented different rootstocks, cultural practices and growing conditions. The generally distinct cluster of points for each cultivar in the correspondence analysis scatterplots indicates that the quantitative polyphenolic profile characteristics are determined principally by the breeding line rather than by growing conditions. Since some apple cultivars are difficult to identify from samples of fruit, quantitative HPLC analysis of the polyphenolic compounds in the fruit with correspondence analysis of the polyphenol profile may be useful in taxonomic studies of apple cultivars for classification and identification.

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## REFERENCES

- Challice J S, Williams A H 1968 Phenolic compounds of the genus *Pyrus*. II. A chemotaxonomic survey. *Phytochemistry (Oxf)* **7** (10) 1781–1801.
- Denford K E 1984 Phytochemical approaches to biosystematics. In: *Plant Biosystematics*, ed Grant W F. Academic Press, New York, pp 359–376.
- Dick A J, Redden P R, DeMarco A C, Lidster P D, Grindley T B 1987 Flavonoid glycosides of Spartan apple peel. *J Agric Food Chem* **35** 529–531.
- Digby P G N, Kempton R A 1987 *Multivariate Analysis of Ecological Communities*. Chapman and Hall, London.
- Greenacre M J 1984 *Theory and Applications of Correspondence Analysis*. Academic Press, London.
- Greenacre M J, Hastie T 1987 The geometric interpretation of correspondence analysis. *J Amer Stat Assoc* **82** 437–447.
- Genstat 1983 A general statistical program, Version 4.04. Numerical Algorithms Group, Oxford.
- Harborne J B 1975 Biochemical systematics of flavonoids. In: *The Flavonoids*, eds Harborne J B, Mabry T J & Mabry H. Academic Press, London, pp 1056–1095.
- Lea A G H 1982 Reversed-phase high-performance liquid chromatography of procyanidins and other phenolics in fresh and oxidising apple juice using a pH shift technique. *J Chromatogr* **238** 253–257.
- Lidster P D, McRae K B, Sanford K A 1981 Responses of McIntosh apples to low oxygen storage. *J Amer Soc Hort Sci* **106** (2) 159–162.
- Stewart R N, Asen S, Massie D R, Norris K H 1980 Identification of poinsettia *Euphorbia pulcherriura* cultivars by high pressure liquid chromatography analysis of their flavonal content. *Biochem Syst Ecol* **8** 119–126.
- Teuber Wuenscher H, Herrmann K 1978 Flavonol glycosides of apples (*Malus silvestris* Mill.) 10. Phenolic contents of fruits, *Z Lebensm-Unters-Forsch* **166** 80–84.
- Underhill L G, Peisach M 1985 Correspondence analysis and its application in multielemental trace analysis. *J Trace Microprobe Tech* **3** (1&2) 41–65.

## **Sensory Study of Flavour Compounds in Extracts of Salted Salmon Eggs (Ikura)**

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### **ABSTRACT**

*Water soluble extracts were prepared from ikura and each component was analysed. Upon reconstruction of a synthetic ikura extract with pure reagents on the basis of the analytical data, flavour components were sensorily examined by an omission test. This revealed that only 12 components played an important role in producing the characteristic taste of ikura. The novelty of some nucleosides as taste factors is also discussed.*

**Key words:** Salmon roe, sensory test, sea food, flavour extractives, nucleoside.

### **INTRODUCTION**

Matured salmon roe is salted, separated into individual eggs and served under the name of ikura as one of the most popular and expensive fish products in Japan (Tanikawa 1971). It has a pleasing aroma characteristic of marine products and is rich in salty, oily and umami (MSG-like meaty) tastes. Ikura can be served as it is or with seasonings such as soy sauce (shoyu) and sweet rice wine (mirin).

An artificial ikura analogue has come on to the market as a response to recent substantial rises in the price of the natural products. This is a hybrid product of newly developed membrane and sophisticated microcapsulation technology. It is made by enveloping vegetable oil in a low-calorie natural gel such as one made with alginic acid. Its texture resembles that of the natural product. No analytical data on flavour components of natural ikura have previously been reported. The manufacturer of the ikura analogue claims to use a mixture of an artificial flavour

and a small portion of natural marine extracts to improve the taste of the ikura analogue.

This paper reports data on water soluble constituents of three brands of natural ikura and the results of a series of sensory tests on an artificial ikura extract formulated on the basis of the analysis.

## EXPERIMENTS

### Materials

About 1-kg samples of each of three different ikura batches were purchased in December 1985 directly from the manufacturer. These comprised (1) S-grade, manufactured in Miyagi Prefecture; its wholesale price was 4500 Yen (\$35)  $\text{kg}^{-1}$ ; (2) A-grade, manufactured in Miyagi Prefecture, 3000 Yen (\$23)  $\text{kg}^{-1}$ ; and (3) S-grade, manufactured in Hokkaido Prefecture, 5500 Yen (\$42)  $\text{kg}^{-1}$ . The samples were kept frozen at  $-25^{\circ}\text{C}$  until analysed.

### Proximate composition

Moisture, crude protein, crude fat and crude ash were determined in duplicate according to AOAC (1980) methods.

### Preparation of extract for chemical analysis

About 100 g of the material was homogenised with ethanol to a final concentration of 700 g  $\text{litre}^{-1}$  and centrifuged. This process was repeated twice and all three supernates were combined and held at  $2^{\circ}\text{C}$  overnight, and the ethanol was evaporated. The concentrate was extracted three times with twice its volume of diethyl ether and the combined ether layers were backwashed with water to recover as much as possible of the water soluble material. The defatted extract and the recovered water soluble material were combined, the ether was evaporated and the volume was made up to 100 ml with water. This solution was dispensed into small vials and held at  $-30^{\circ}\text{C}$  until analysed.

### Chemical analysis

#### *Free amino acids*

The sample was diluted fivefold with water, filtered through a membrane (pore size  $0.45\ \mu\text{m}$ ) and analysed (Hitachi 835 amino acid analyser) by six-stepwise elution with lithium citrate buffers for physiological fluid analysis (Konosu *et al* 1978). The results were expressed as  $\text{mg kg}^{-1}$  fresh weight.

#### *Combined amino acids*

The data for combined amino acids were obtained by difference between the free figure and that obtained by analysis of a sample hydrolysed with 6 M HCl at  $105^{\circ}\text{C}$  for 18 h.

#### *Nucleotides*

The sample extract was diluted tenfold with water and passed through a membrane

filter (0.45  $\mu\text{m}$  pore size). Nucleotides were separated from each other by Shimadzu LC-6A HPLC on a Shimadzu anion-exchange column Shim-pack WAX (4 mm  $\times$  50 mm) with a 50 mM (pH 7.0) to 480 mM (pH 6.0) linear gradient phosphate buffer, detected at 260 nm using a Shimadzu SPD-6A UV monitor, and calculated automatically with a Shimadzu C-R3A data processor.

#### *Nucleosides and nucleic bases*

The extract was pretreated by passing it through a Dowex 1-X4 column to remove nucleotides. After concentration by evaporation under reduced pressure, it was chromatographed isocratically on a reversed phase column Shimadzu Shim-pack CLC-ODS (6 mm  $\times$  150 mm) with 0.2 M sodium perchlorate in 0.1 M phosphate buffer (pH 2.0). Monitoring and integration were carried out by the same system as used for the determination of nucleotides.

#### *Organic bases*

Betaines in the extract were converted to *p*-bromophenacyl esters by treatment with *p*-bromophenacyl bromide in the presence of crown ether (18-crown-6; Merck) and  $\text{KH}_2\text{PO}_4$  (Konosu *et al* 1986). They were then separated on a Whatman Partisil 10-SCX column using 30–50  $\mu\text{M}$   $\text{KH}_2\text{PO}_4$  in aqueous methanol (20 ml methanol litre<sup>-1</sup>) and detected at 262 nm. TMAO in the extract was first reduced to TMA by addition of 1 ml freshly prepared titanium trichloride (100 g litre<sup>-1</sup>) and allowing the solution to stand for 2 h at room temperature. The excess reducing agent was neutralised with two to three drops of saturated potassium carbonate solution. The TMA was determined by Shimadzu GC-7A GC using a 3 mm  $\times$  2 m glass column packed with squalene (20 g):glycerol (2.5 g):KOH (2.5 g) on Diasolid L (150–170  $\mu\text{m}$  dia, 75 g).

#### *Organic acid*

The extract was loaded on to an Amberlite IRA-45 and washed with deionised water. Then another column of Dowex 50W-X8 was connected to the Amberlite column and the organic acid fraction was eluted with 1 M NaOH. After making up to appropriate volume with water it was analysed by HPLC on the Shimadzu LC-6A equipped with a Shim-pack SCR 101H (7.9 mm  $\times$  300 mm) column and Shimadzu SPD-6A UV monitor. The compounds were eluted with deionised water adjusted to pH 2.1 with perchloric acid. Each peak area was integrated and calculated automatically with the Shimadzu C-R3A and expressed as mg kg<sup>-1</sup> fresh weight.

#### *Sugar*

After confirmation that the pH of the test solution was neutral, simple sugars were reduced to the corresponding sugar alcohols with an equal volume of aqueous sodium borohydride (10 g litre<sup>-1</sup>) at room temperature for 1 h. Residual reducing agent was destroyed by addition of glacial acetic acid and the solution was treated three times with a mixture of hydrochloric acid and methanol. After being dried it was dissolved in an equal amount mixture of pyridine and acetic anhydride. A small portion of the reactant was chromatographed isothermally at 225°C on the

Shimadzu GC-7A using a glass column of 3 mm  $\times$  2.1 m packed with GP 3% SP-2340 on Supelcoport (150–170  $\mu$ m dia). The injection temperature was 260°C and the carrier gas flow rate was 40 ml min<sup>-1</sup> (0.7 kg cm<sup>-2</sup>). All data were calculated as mg kg<sup>-1</sup> raw material.

#### *Inorganic ions*

Concentrations of six cations (Na, K, Mg, Ca, Cu and Cd) were determined by atomic absorption analysis using a Japan Jarrell Ash AA-860 (Kyoto). Chloride was determined by Mohr's method and phosphoric ion using ammonium molybdate reagent. The results were calculated as mg kg<sup>-1</sup> raw material.

#### **Preparation of test solutions**

##### *Completely reconstructed extract*

The completely reconstructed extract was formulated from commercially available analytical grade reagents as shown in Table 7 on the basis of analyses of the natural extract prepared from Miyagi's S-grade material.

##### *Reconstructed extracts for omission tests*

Reconstructed extracts of a different formulation were prepared as for the completely reconstructed extract but omitting either a group of components or a single component.

#### **Sensory assessment**

The sensory panel (five men and one woman in the laboratory) was trained to the standard level of proficiency of institutional taste panellists (Furukawa 1977). In the first step of the sensory analysis, the most suitable concentration was examined by open panel discussion to detect the fine differences between the test solutions lacking one or more constituents. Then the reconstructed ikura extract diluted to a suitable concentration with water was subjected to a triangle difference test by the panellists acting independently. In the omission tests, a set of test solutions at room temperature (one different and two the same) was given to each panellist. The panellists were asked to point out the odd sample. Each assessment was repeated and all 12 responses were statistically processed using the table of criteria for the triangle difference test. The panellists were also requested to describe the difference in taste profile between the two test samples presented.

## **RESULTS AND DISCUSSION**

#### **General composition of ikura**

The sum of moisture, crude protein, crude fat and crude ash of any ikura examined reached only about 90%, although carbohydrates were not measured individually. This must be a peculiarity of salted fish eggs. Fish roe lipid generally contains a large quantity of phospholipids which are difficult to extract by the ordinary Soxhlet method with diethyl ether. However, a low recovery does not seem to interfere with comparison of the quality of ikura samples. Both Miyagi and Hokkaido S-grade

**TABLE 1**  
Proximate composition of test materials (g kg<sup>-1</sup>)

| Name                | Moisture | Crude protein | Crude fat | Crude ash | Total |
|---------------------|----------|---------------|-----------|-----------|-------|
| Ishi-S <sup>a</sup> | 476      | 316           | 90        | 37        | 919   |
| Ishi-A <sup>b</sup> | 509      | 293           | 62        | 35        | 899   |
| Hokk-S <sup>c</sup> | 427      | 314           | 90        | 41        | 872   |

<sup>a</sup> Ishi-S: Ikura, S-grade, manufactured in Ishinomaki, Miyagi Prefecture.

<sup>b</sup> Ishi-A: Ikura, A-grade, manufactured in Ishinomaki, Miyagi Prefecture.

<sup>c</sup> Hokk-S: Ikura, S-grade, manufactured in Hokkaido Prefecture.

ikura showed slight differences in water content (Table 1). Miyagi A-grade ikura contained less crude fat and more water than the others.

### Taste profile of ikura

Open panel discussion by six members revealed rich sweetness, saltiness and umami (MSG-like meaty) taste, with stickiness in Hokkaido S-grade. However, some panellists considered the latter to have too salty a taste. Miyagi S-grade had moderate saltiness and gained a high overall score in open panel discussion. On the other hand, Miyagi A-grade was given a lower score on account of both a slightly harder egg membrane texture, which resulted in adherence to the teeth, and a watery taste.

### Analysis of extractive components

#### Free amino acids in the extract

The total free amino acids in the extract (Table 2) amounted to 0.7–1.5 g kg<sup>-1</sup> raw material. This corresponds to about 20–40% of the amount in salmon (Shirai *et al* 1983) and yellow tail (Murata and Sakaguchi 1988) muscles. Of 29 amino acids and their related compounds, Tau, Asp, Ser, Glu and Leu (see footnote to Table 2 for key to less common amino acids) were the major ones and their contents were ~10 mg or less. They comprised about half of the total amino acids. The level of Glu, the amino acid present in the largest amount in both Miyagi S- and A-grades, might suggest the exogenous influence of the condiment usually rich in MSG.

#### Combined amino acids in the extract

Upon acid hydrolysis of the extracts, total amino acids increased 35, 74, and 313% in Miyagi S- and A-grade and Hokkaido S-grade, respectively (Table 2). This suggests that the extract of Hokkaido S-grade might be abundant in peptides which are soluble in 70% ethanol. Glu, Leu, Ser, and Asp were responsible for more than 50% of the overall increase.

#### Nucleotide in the extract

Analytical results are summarised in Table 3. IMP, ranging from 6 to 18 mg kg<sup>-1</sup> raw material, was the only nucleotide detected in all three extracts analysed. It is the major nucleotide in fish muscle thought to be responsible for its flavour (Murata

TABLE 2  
Free and combined<sup>a</sup> amino acids composition of ikura extract (mg kg<sup>-1</sup>)

| Amino acid                 | Ishi-S    | Ishi-A    | Hokk-S   | Amino acid                 | Ishi-S     | Ishi-A     | Hokk-S     |
|----------------------------|-----------|-----------|----------|----------------------------|------------|------------|------------|
| Gau <sup>b</sup>           | 136 (44)  | 157 (—)   | 199 (—)  | Ile                        | 86 (3)     | 48 (16)    | 20 (115)   |
| Asp                        | 124 (47)  | 103 (41)  | 21 (213) | Leu                        | 117 (9)    | 63 (190)   | 25 (525)   |
| Thr                        | 66 (—)    | 43 (10)   | 31 (66)  | Tyr                        | 56 (5)     | 38 (3)     | 36 (35)    |
| Ser                        | 140 (98)  | 77 (109)  | 15 (267) | Phe                        | 76 (—)     | 43 (4)     | 45 (61)    |
| Asn <sup>b</sup>           | — (—)     | 11 (—)    | — (—)    | $\beta$ -Ala               | — (14)     | — (19)     | 2 (7)      |
| Glu                        | 186 (126) | 177 (183) | 34 (375) | $\beta$ -AiBA <sup>b</sup> | — (7)      | — (8)      | — (11)     |
| Sar <sup>b</sup>           | 3 (—)     | — (—)     | 6 (—)    | $\gamma$ -ABA <sup>b</sup> | 2 (—)      | 1 (—)      | 1 (—)      |
| $\alpha$ -AAA <sup>b</sup> | 6 (—)     | 3 (2)     | 5 (2)    | EA <sup>b</sup>            | 23 (7)     | 12 (—)     | 16 (—)     |
| Pro                        | 32 (18)   | 11 (17)   | 28 (39)  | NH <sub>3</sub>            | 15 (59)    | 19 (61)    | 77 (6)     |
| Gly                        | 40 (54)   | 28 (—)    | 25 (—)   | Orn <sup>b</sup>           | 2 (5)      | 5 (7)      | 4 (4)      |
| Ala                        | 89 (33)   | 63 (40)   | 10 (156) | Lys                        | 90 (—)     | 36 (9)     | 38 (96)    |
| Cit <sup>b</sup>           | 1 (—)     | 3 (—)     | — (—)    | 1-MeHis <sup>b</sup>       | — (16)     | — (—)      | — (—)      |
| $\alpha$ -ABA <sup>b</sup> | — (4)     | 1 (3)     | — (3)    | His                        | 21 (4)     | 11 (4)     | 8 (28)     |
| Val                        | 96 (3)    | 59 (19)   | 39 (110) | Ans <sup>b</sup>           | — (—)      | — (—)      | — (—)      |
| Cys                        | — (24)    | — (20)    | — (34)   | Car <sup>b</sup>           | 28 (—)     | — (—)      | — (—)      |
| Met                        | 31 (1)    | 20 (9)    | 14 (33)  | Arg                        | 66 (2)     | 33 (14)    | 3 (103)    |
| Cysthi <sup>b</sup>        | 2 (—)     | — (—)     | 1 (—)    | Total                      | 1534 (583) | 1065 (788) | 703 (2289) |

<sup>a</sup> Combined amino acid. The amount by which the figure for free amino acids increased after acid hydrolysis is given in parentheses.

<sup>b</sup> Tau, taurine; Asn, asparagine; Sar, sarcosine;  $\alpha$ -AAA,  $\alpha$ -aminoadipic acid; Cit, citrulline,  $\alpha$ -ABA,  $\alpha$ -aminobutyric acid; Cysthi, cystathionine;  $\beta$ -AiBA,  $\beta$ -aminoiso-butyric acid;  $\gamma$ -ABA,  $\gamma$ -aminobutyric acid; EA, ethanolamine; Orn, ornithine; 1-MeHis, 1-methylhistidine; Ans, anserine; Car, carnosine.

— Not detected; + trace.

**TABLE 3**  
Nucleotide and related compounds in ikura extract (mg kg<sup>-1</sup>)

| Nucleotide   | Ishi-S | Ishi-A | Hokk-S |
|--------------|--------|--------|--------|
| IMP          | 18     | 13     | 6      |
| AMP          | —      | 12     | 18     |
| GMP          | —      | 4      | —      |
| Inosine      | 78     | 39     | 20     |
| Adenosine    | 42     | 47     | 59     |
| Guanosine    | 43     | 15     | 20     |
| Uridine      | 51     | 24     | 77     |
| Hypoxanthine | 34     | 32     | 33     |
| Uracil       | 31     | 36     | 30     |
| Cytosine     | 251    | 257    | 301    |

— Not detected.

**TABLE 4**  
Organic base composition in ikura extract (mg kg<sup>-1</sup>)

| Base                      | Ishi-S | Ishi-A | Hokk-S |
|---------------------------|--------|--------|--------|
| TMAO                      | 623    | 640    | 521    |
| TMA                       | 17     | 21     | 21     |
| Glycine betaine           | —      | —      | 180    |
| $\beta$ -Alanine betaine  | —      | —      | —      |
| $\gamma$ -Butyrol betaine | —      | —      | —      |

— Not detected.

and Sakaguchi 1986), and is a useful index of freshness (Surette *et al* 1988). AMP and GMP (*c* 10 mg kg<sup>-1</sup>) were also determined in some samples. No CMP, which was found to be present in a considerable amount in crab ovary (Hayashi *et al* 1978), was detected in any ikura extract.

#### *Nucleosides and nucleic bases in the extract*

Four nucleosides were found in the extract, ranging from 15 to 78 mg kg<sup>-1</sup> raw material. There were no big differences between samples tested. Cytosine was the major component present at around 300 mg kg<sup>-1</sup> and its amount was about ten times higher than that of the two other nucleic bases detected. However, cytidine, the nucleoside corresponding to cytosine, was not detected.

#### *Organic bases in the extract*

No betaines were detected except for glycine betaine in Hokkaido S-grade (Table 4). TMAO, which plays an important role in osmoregulation (Barns and Blackstock 1974), was detected in all extracts at about 600 mg kg<sup>-1</sup>. This seems the typical concentration for extracts prepared from viscera of marine animals. Very small amounts of TMA were found in all extracts tested.

TABLE 5  
Non-nitrogenous compounds in ikura extract (mg kg<sup>-1</sup>)

| Compound     | Ishi-S | Ishi-A | Hokk-S |
|--------------|--------|--------|--------|
| Oxalic acid  | 2      | —      | —      |
| Citric acid  | 5      | 4      | —      |
| Fumaric acid | —      | +      | —      |
| Glucose      | 84     | 88     | 131    |

— Not detected; + trace.

TABLE 6  
Inorganic ions in ikura extract

| Ion             | Units               | Ishi-S | Ishi-A | Hokk-S |
|-----------------|---------------------|--------|--------|--------|
| Na              | g kg <sup>-1</sup>  | 11.65  | 4.47   | 3.70   |
| K               | g kg <sup>-1</sup>  | 1.91   | 2.46   | 2.34   |
| Cl              | g kg <sup>-1</sup>  | 10.55  | 13.43  | 13.89  |
| Mg              | mg kg <sup>-1</sup> | 48     | 49     | 47     |
| Ca              | mg kg <sup>-1</sup> | 3      | 4      | 5      |
| Cu              | mg kg <sup>-1</sup> | —      | —      | —      |
| PO <sub>4</sub> | mg kg <sup>-1</sup> | 333    | 317    | 298    |

— Not detected.

*Organic acids in the extract*

Out of nine organic acids looked for, only oxalic, citric and fumaric acids were found in some extracts (Table 5). The distribution of organic acids in marine fish and invertebrates was studied by Osada (1966) who found that lactic and succinic acids were the major ones; neither was detected in ikura extracts.

*Sugars in the extract*

Glucose was the only sugar detected in ikura extract at around 100 mg kg<sup>-1</sup> raw material (Table 5).

*Inorganic ions in the extract*

As in many marine products (Lauer *et al* 1974; Resources Council Japan 1982), sodium (3.70–11.65 g kg<sup>-1</sup>) and potassium (1.91–2.46 g kg<sup>-1</sup>) ions comprised the major part of the minerals in ikura extract (Table 6). The contents of magnesium and calcium were found to be only a few milligrams. The chloride content was around 13.0 g kg<sup>-1</sup>. The amount of phosphate ion in every extract tested was about 0.3 g kg<sup>-1</sup>.

**Comparison of the flavour profiles of natural and reconstructed extracts**

Upon open panel discussion, all panellists pointed out the lack of characteristic oily flavour in the natural extract because of the defatting process used in its preparation. However, very little difference was recorded between natural and

reconstructed extracts in the taste profiles. Thus, the reconstructed extract was adopted as a reference standard in the sensory analysis since the (completely) reconstructed extract reproduced adequately the taste of natural ikura extract.

### Group omission test

The 36 constituents given in Table 7 were divided into six groups in order to carry out the group omission tests. They were amino acids, nucleotides, organic bases, organic acids, sugars and minerals. The reconstructed extract was diluted to 70% of the original concentration.

The results of the group omission test (Table 8) indicate that any one of the six groups contributed to the ikura taste to some extent. Inorganic ions showed the highest level of significance in the omission test, suggesting the importance of their role in the characteristic ikura taste. Besides this, glucose, the only sugar included in the extract, also contributed to the ikura taste though weakly.

In the second stage of the group omission test, groups which contained more than three components were divided into a few subgroups. Amino acids were divided into acidic, neutral, basic and 'other' amino acids. Among these four subgroups, acidic and 'other' amino acids were found to contribute to the essence of ikura flavour. Nucleotides were split into nucleotide (IMP only), nucleosides, and nucleic bases. All three groups contributed to the flavour. Besides IMP, each component of the remaining two subgroups was eliminated consecutively from the reconstructed extract. Inosine and adenosine, and guanosine, were shown to be significant in ikura flavour at 5% and 0.1% level of confidence, respectively. The second step of the sensory test is summarised in Table 9 together with the results of individual omission. Each single constituent of a taste-active subgroup was omitted in turn. Among the amino acids, only Glu was found to be essential at the 99% level of significance to produce the characteristic ikura flavour.

In conclusion, Glu, IMP, inosine, adenosine, guanosine, uracil, TMA, glucose and four kinds of inorganic ion were revealed to contribute to the characteristic ikura flavour by a series of sensory tests summarised in Fig 1. Besides these 12 constituents, Tau and Pro, or oxalic and succinic acids, also contributed to the ikura flavour when two of them co-existed in the extract. In the case of the earlier study in this laboratory on boiled crab meat flavour, both Tau and Pro were found in large amounts ( $2.0\text{--}3.0\text{ g kg}^{-1}$ ) although they showed no contribution to the crab flavour when they were omitted individually (Hayashi *et al* 1981). The amounts of these two amino acid related compounds were  $140$  and  $30\text{ mg kg}^{-1}$  raw material, respectively, in ikura extract and were less than one-tenth of those in the crab extract. Thus it is possible that a synergistic effect occurs between Tau and Pro at these low concentrations. The sum of the weights of the two organic acids was  $7\text{ mg kg}^{-1}$  which comprised only 0.03% of the total weight of reconstructed extract constituents ( $2.23\text{ g}$ ) yet they none the less contributed to the taste of ikura. These results suggest the complexity of the role of each water-soluble component in producing the complicated and attractive food flavour when more than three components exist together in the same system.

The well known synergistic taste effect between Glu and IMP was confirmed in this study. Both of them constitute the major factor in the ikura taste of water-

TABLE 7  
Composition of completely reconstructed ikura extract

| Amino acid (mg kg <sup>-1</sup> ) |     | Nucleotide (mg kg <sup>-1</sup> ) |     | Organic base (mg kg <sup>-1</sup> ) |     | Mineral (g kg <sup>-1</sup> )       |     |                                 |       |
|-----------------------------------|-----|-----------------------------------|-----|-------------------------------------|-----|-------------------------------------|-----|---------------------------------|-------|
| Tau <sup>a</sup>                  | 136 | Met                               | 31  | IMP <sup>b</sup>                    | 27  | TMAO                                | 623 | KCl                             | 3.64  |
| Asp                               | 124 | Ile                               | 86  | Nucleoside (mg kg <sup>-1</sup> )   | 51  | TMA                                 | 17  | NaCl                            | 14.54 |
| Thr                               | 66  | Leu                               | 117 |                                     |     |                                     |     | Na <sub>3</sub> PO <sub>4</sub> | 1.32  |
| Ser                               | 140 | Tyr                               | 56  | Inosine                             | 78  |                                     |     |                                 |       |
| Glu <sup>c</sup>                  | 235 | Phe                               | 76  | Adenosine                           | 42  | Organic acid (mg kg <sup>-1</sup> ) |     |                                 |       |
| $\alpha$ -AAA                     | 6   | Lys                               | 90  | Guanosine                           | 43  | Oxalic acid                         | 2   |                                 |       |
| Pro                               | 32  | His                               | 21  | Nucleic base (mg kg <sup>-1</sup> ) | 251 | Citric acid                         | 5   |                                 |       |
| Gly                               | 40  | Car                               | 28  |                                     |     |                                     |     |                                 |       |
| Ala                               | 89  | Arg                               | 66  | Uracil                              | 31  | Sugar (mg kg <sup>-1</sup> )        |     |                                 |       |
| Val                               | 96  |                                   |     | Hypoxanthine                        | 34  | Glucose                             | 84  |                                 |       |

pH 6.0.

<sup>a</sup> See Table 2 for footnote explaining abbreviations.<sup>b</sup> IMP as IMP · 2Na · 7.5H<sub>2</sub>O.<sup>c</sup> Glu as Glu · Na · H<sub>2</sub>O.

**TABLE 8**  
Group omission test

| <i>Omitted group</i> | <i>Number of panellists</i> | <i>Number of correct identifications</i> | <i>Level of significance</i> |
|----------------------|-----------------------------|--|------------------------------|
| Amino acids          | 19                          | 13                                       | **                           |
| Nucleotides          | 19                          | 13                                       | **                           |
| Organic bases        | 19                          | 13                                       | **                           |
| Organic acids        | 15                          | 9  | *                            |
| Sugar                | 19                          | 13                                       | **                           |
| Minerals             | 13                          | 12                                       | ***                          |

\*\*\* $P < 0.001$ ; \*\* $P < 0.01$ ; \* $P < 0.05$ .

**TABLE 9**  
Subgroup and individual omission test

| <i>Omitted compounds</i> | <i>Number of panellists</i> | <i>Number of correct identifications</i> | <i>Level of significance</i> |
|--------------------------|-----------------------------|--|------------------------------|
| Acidic amino acids       | 8                           | 7  | **                           |
| Asp                      | 18                          | 8  | —                            |
| Glu                      | 18                          | 12                                       | **                           |
| Neutral amino acids      | 8                           | 3  | —                            |
| Basic amino acids        | 8                           | 4  | —                            |
| Others (Tau, Pro)        | 8                           | 6  | *                            |
| Tau                      | 18                          | 7  | —                            |
| Pro                      | 18                          | 7  | —                            |
| Nucleotide (IMP only)    | 12                          | 9  | **                           |
| Nucleosides              | 12                          | 8  | *                            |
| Inosine                  | 18                          | 10                                       | *                            |
| Adenosine                | 18                          | 10                                       | *                            |
| Uridine                  | 18                          | 9  | —                            |
| Guanosine                | 18                          | 13                                       | ***                          |
| Nucleic free bases       | 12                          | 12                                       | ***                          |
| Uracil                   | 18                          | 17                                       | ***                          |
| Hypoxanthine             | 18                          | 6  | —                            |
| Organic bases            |                             |  |                              |
| TMA                      | 12                          | 9  | **                           |
| TMAO                     | 12                          | 6  | —                            |
| Organic acids            |                             |  |                              |
| Oxalic acid              | 12                          | 4  | —                            |
| Citric acid              | 12                          | 4  | —                            |

\*\*\* $P < 0.001$ ; \*\* $P < 0.01$ ; \* $P < 0.05$ ; — Not significant.

soluble components. Among the related nucleotide compounds, three nucleosides and one free nucleic base were shown to play an important role in producing the characteristic ikura taste. Of five nucleosides and nucleic bases, which are normally found in marine animal body fluids, only hypoxanthine was reported to contribute to bitterness in cod muscle (Jones 1960). Very few papers have reported on the taste of these compounds in marine products. In the present series of sensory tests, the

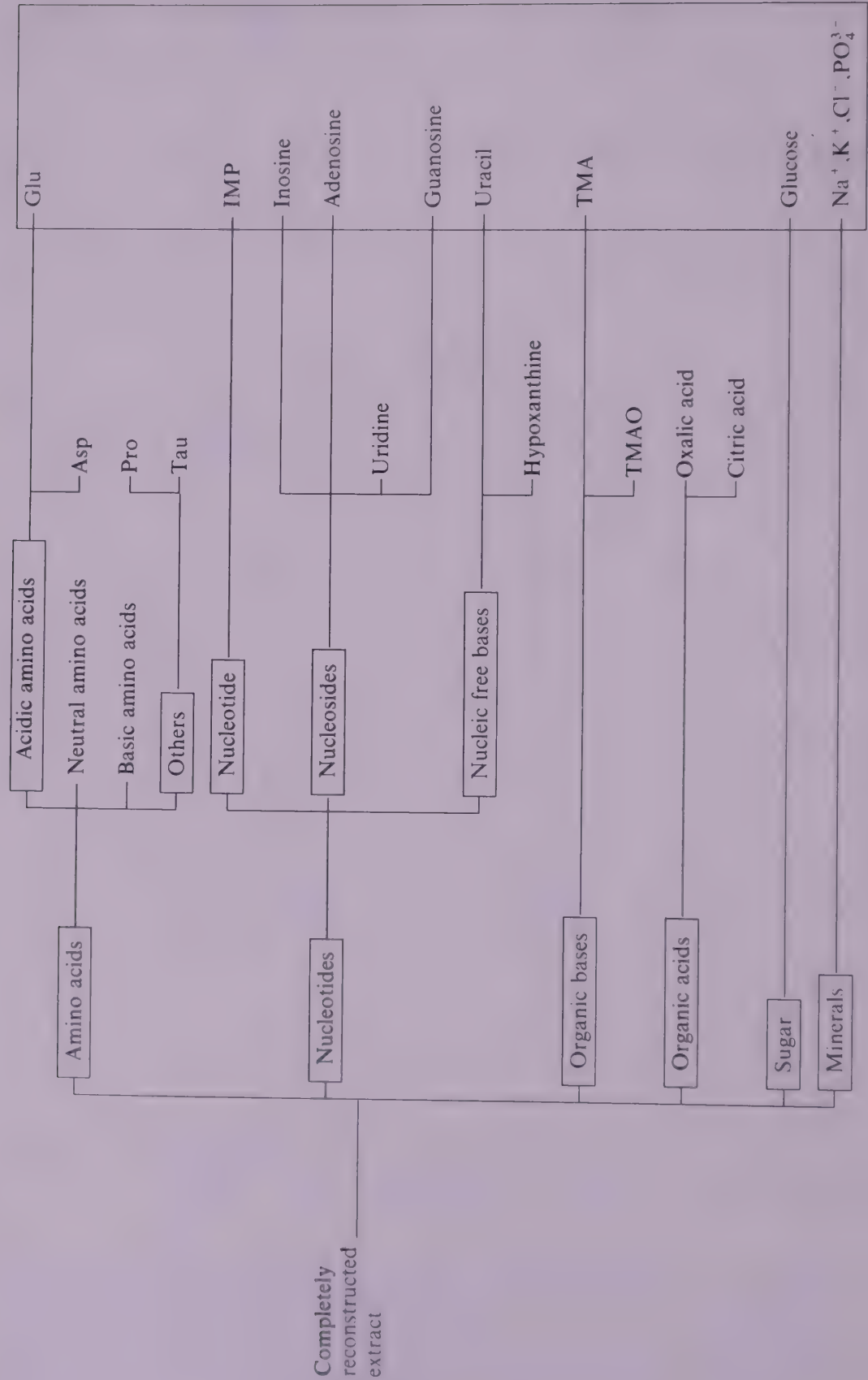


Fig 1. Summary of sensory omission test. The components enclosed in boxes were judged to contribute to the characteristic taste of ikura.

panel distinguished the difference in taste between completely reconstructed extract and extract from which one of inosine, adenosine, guanosine or uracil was omitted. However, at the same time, most panellists declared difficulty in explaining the nature of the difference by the customary sensory terms such as sweetness, saltiness, bitterness, sourness or umami. Further examination of this subject may be required to make clear the role of these four compounds in producing the unique taste of ikura.  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Cl}^-$  and  $\text{PO}_4^{3-}$  were included in the completely reconstructed extract, since it was difficult to study separately (Hayashi *et al* 1981) the role of each of the inorganic ions in producing the characteristic ikura flavour. In contrast to other water soluble components, omission of one ion influenced the effect of remaining ions and it was very difficult to analyse quantitatively their contribution to the taste. Not only because of their high concentration (84% w of the total constituents of completely reconstructed extract) but also because of the fundamental role of chloride and sodium ions as taste stimuli to the neurotransmitting system, inorganic ions are considered to be indispensable to the characteristic flavour of ikura.

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### REFERENCES

- Barnes H, Blackstock J 1974 The separation and estimation of free amino acids, trimethylamine oxide, and betaine in tissues and body fluids of marine invertebrates. *J Exp Mar Biol Ecol* **16** 29–45.
- Furukawa H 1977 Selection of panelists for organoleptic test. *Proc 7th Symp Sensory Inspection*. Union Japan Scientists Engineers, Tokyo, pp 111–121.
- Hayashi T, Yamaguchi K, Konosu S 1981 Sensory analysis of taste-active components in the extract of boiled snow crab meat. *J Food Sci* **46** 479–483 and 493.
- Jones N R 1960 The separation and determination of free purines, pyrimidines and nucleoside in cod muscle. *Analyst* **85** 111–115.
- Konosu S, Yamaguchi K, Hayashi T 1978 Studies on flavor components in boiled crabs—I Amino acids and related compounds in the extracts. *Bull Japan Soc Sci Fish* **44** 505–510.
- Konosu S, Shinagawa A, Yamaguchi K 1986 Determination of omega-betaines in aquatic animals by HPLC. *Bull Japan Soc Sci Fish* **52** 869–873.
- Lauer B H, Murray M C, Anderson W E, Guptill E B 1974 Atlantic queen crab, jonah crab and red crab. Proximate composition of crabmeat from edible tissues and concentration of some major mineral constituents in the ash. *J Food Sci* **39** 383–385.
- Murata M, Sakaguchi M 1986 Storage of yellowtail (*Seriola quinqueradiata*) white and dark muscle in ice: changes in content of adenine nucleotides and related compounds. *J Food Sci* **51** 321–326.
- Murata M, Sakaguchi M 1988 Changes in free amino acids and adenine nucleotides in boiled muscle extracts of yellowtail (*Seriola quinqueradiata*) stored in ice. *J Agric Food Chem* **36** 595–599.

- Osada H 1966 Studies on the organic acids in marine products—I: distribution of the organic acids in marine products. *J Toyo Jun Col* **7** 271–274.
- Resources Council Japan 1982 Fishes and shellfishes. *Std Tab Food Comp Japan*. Resources Council Japan, Tokyo, pp 104–151.
- Shirai T, Fuke S, Yamaguchi K, Konosu S 1983 Studies on extractive components of salmonids—II Comparison of amino acids and related compounds in the muscle extracts of four species of salmon. *Comp Biochem Physiol* **74B** 685–689.
- Surette M E, Gill T A, LeBlanc P J 1988 Biochemical basis of postmortem nucleotide catabolism in cod (*Gadus morhua*) and its relationship to spoilage. *J Agric Food Chem* **36** 19–22.
- Tanikawa E 1971 (ed) Fish salting industry. In: *Marine Products in Japan*. Koseisha-Koseikaku, Tokyo, pp 295–313.

## **Volatile Flavour Constituents of Feijoa (*Feijoa sellowiana*)—Analysis of Fruit Flesh**

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### **ABSTRACT**

*The volatile flavour fraction from the flesh of ripe feijoa fruit (Feijoa sellowiana Berg cv Mammoth) has been isolated by high vacuum steam distillation and subsequent liquid–liquid extraction of the volatiles. Analysis by capillary GC and GC–MS led to the identification of 15 constituents of which (Z)-hex-3-enal and isopropyl benzoate are reported for the first time. Methyl benzoate constituted 82% of the volatile flavour extract.*

**Key words:** Feijoa, *Feijoa sellowiana* Berg, fruit flesh, volatile flavour constituents, GC, GC–MS.

### **INTRODUCTION**

The volatile constituents of feijoa have attracted wide attention due to the distinctive fruity character of this fruit and because high quality feijoa are popular as a dessert (Bollard 1981). The first study of the flavour volatiles in feijoa was reported by Hardy and Michael (1970). These authors identified 16 components from a vacuum steam distillate of whole fruit. Methyl and ethyl benzoate accounted for 90% of the volatile fraction and were both considered to be primarily responsible for the strong 'feijoa-like' character of the fruit.

In a comprehensive investigation, Shiota *et al* (1980) identified 57 components in New Zealand grown feijoa and confirmed the presence of all but heptan-2-one from the earlier study. Of these, octan-3-one, methyl benzoate, ethyl benzoate and linalool comprised 70% of the volatile fraction from the whole fruit.

As a result of the increased interest in assessing the commercial potential of feijoa fruit for the fresh fruit market and for opportunities in downstream processing, there has been a need for more information on the volatile flavour profiles from different parts of the feijoa as opposed to whole fruit analysis. Accordingly the authors initiated a reinvestigation of previous studies by reporting (Shaw *et al* 1983) on the major volatile flavour constituents emanating from intact fruit and the quantitative changes in the flavour profile at different stages of ripeness.

The present paper provides information on the isolation and characterisation of the principal volatile flavour constituents contributing to the characteristic aroma of ripe feijoa flesh, and assesses these results in the light of data presented in other studies.

## EXPERIMENTAL

### Plant material

Feijoa fruit (*Feijoa sellowiana* Berg cv Mammoth) were obtained from a local orchard. Fruit that had been naturally abscised over a 24-h period were collected and stored at 18–20°C until judged ripe (8 days). Three representative samples of approximately 5 kg each were selected for analysis.

### Isolation and concentration of volatile flavour constituents

Each fruit was sliced in half and the soft flesh was removed. The sample was pureed in 4 litres of deionised glass-distilled water using a Waring blender and then vacuum steam distilled at 30–35°C. The steam distillate (500 ml) was collected over 3 h in a 2-litre round-bottomed flask cooled with solid carbon dioxide/isopropanol, and then continuously extracted with double-distilled diethyl ether for 3 h. The extract (200 ml) was dried over anhydrous magnesium sulphate for 12 h before being filtered and carefully concentrated to approximately 5 ml using a low-hold-up Vigreux column (Quickfit, Stone, Staffs, UK). The concentrate was sealed in a small glass vial and held at –10°C until required.

### Gas chromatography

Analyses of the feijoa concentrates were carried out on a Hewlett-Packard 5840A GC equipped with a Grob splitless injector and FID detector. Separations were performed using (a) a 50 m × 0.2 mm id WCOT glass capillary column coated with Carbowax 20M, and (b) a 50 m × 0.2 mm id fused silica capillary column coated with OV-101. Each column was temperature programmed from 50 to 200°C at 4°C min<sup>-1</sup> and held at the maximum temperature for 20 min; nitrogen carrier gas 1 ml min<sup>-1</sup>; injector, 220°C; detector, 230°C.

### Gas chromatography odour assessment

Odour assessments were carried out using a Hewlett Packard 5760A GC as described previously (Shaw *et al* 1985).

### Gas chromatography-mass spectrometry

Low resolution electron impact GC-MS analysis of the volatile flavour concentrates were performed using either (a) a Kratos MS30 GC-MS, or (b) a Hewlett-Packard 5885A GC-MS. Instrument operating conditions have been described previously (Shaw *et al* 1985).

## RESULTS AND DISCUSSION

For the purposes of this investigation only fruit that had been stored at ambient temperature for 8 days after natural abscission were analysed. The volatile flavour fraction from three bulked samples of ripe feijoa flesh was isolated by low-temperature vacuum steam distillation and subsequent liquid-liquid extraction with diethyl ether. In each case the extract, on concentration, had a typically distinctive but strong 'green/fruity' odour, reminiscent of freshly sliced feijoa, due to the presence of ethyl butanoate; ethyl benzoate and methyl benzoate (Shaw *et al* 1983).

A comparison of each capillary GC volatile flavour profile analysed on a 50-m glass Carbowax 20M WCOT column revealed that there was only a 5% variation in the relative amounts of each constituent. Table 1 lists the 15 constituents identified in this investigation together with the average relative abundance of each constituent. Chemical characterisation was primarily by GC-MS with confirmation coming from GC peak enhancement analysis on both Carbowax 20M and OV-101 columns using authentic standards.

Assessment of the odour from the GC effluent indicated that peak 3 had a 'green' character normally associated with C-6 unsaturated aldehydes. The constituent was identified as (Z)-hex-3-enal following synthesis and comparative analysis of both the *cis* and *trans* geometric isomers. The electron impact mass spectra obtained for both isomers under GC-MS operating conditions were essentially identical. However, both isomers were distinguishable by GC (*trans*,  $R_t$  10.7 min; *cis*,  $R_t$  10.9 min) on Carbowax 20M, confirming for the first time the existence of (Z)-hex-3-enal in feijoa fruit. The unequivocal identification of (Z)-hex-3-en-1-ol, (Z)-hex-3-enyl acetate, (Z)-hex-3-enyl butanoate and (Z)-hex-3-enyl benzoate was confirmed in a similar manner.

Sensorially, aliphatic and aromatic esters are considered to be the most important chemical class found either in the whole fruit extract (Hardy and Michael 1970) or in the volatile flavour from intact fruit (Shaw *et al* 1983). In this study a diverse collection of esters constituted the major portion of the extract (85% peak area) with the main contribution coming from aromatic esters. Indeed, the dominating feature of the GC profile was the relatively high concentration of methyl benzoate (82% peak area) which was in agreement with most other previous investigations (Hardy and Michael 1970; Shaw *et al* 1983). Shiota *et al* (1980) on the

TABLE 1  
Volatile flavour constituents in feijoa fruit flesh

| Peak<br>No | Constituent              | Retention time (min) |        | Relative<br>abundance<br>(%) |
|------------|--------------------------|----------------------|--------|------------------------------|
|            |                          | CW20M                | OV-101 |                              |
| 1          | Ethyl butanoate          | 7.9                  | 11.7   | 0.3                          |
| 2          | Hexanal                  | 9.1                  | 11.6   | 0.5                          |
| 3          | (Z)-Hex-3-enal           | 10.9                 | 12.9   | 2.2                          |
| 4          | (E)-Hex-2-enal           | 13.9                 | 13.2   | 1.7                          |
| 5          | (E)- $\beta$ -Ocimene    | 15.4                 | 20.1   | 1.3                          |
| 6          | (Z)-Hex-3-enyl acetate   | 18.5                 | 18.3   | 0.7                          |
| 7          | Hexan-1-ol               | 20.0                 | 13.9   | 0.2                          |
| 8          | (Z)-Hex-3-en-1-ol        | 21.8                 | 13.5   | 2.2                          |
| 9          | Octan-3-ol               | 24.5                 | 19.0   | 0.2                          |
| 10         | (Z)-Hex-3-enyl butanoate | 26.6                 | 24.4   | 0.4                          |
| 11         | Methyl benzoate          | 36.7                 | 21.5   | 81.9                         |
| 12         | Isopropyl benzoate       | 38.4                 | 29.1   | 0.5                          |
| 13         | Ethyl benzoate           | 39.0                 | 24.0   | 0.5                          |
| 14         | Methyl anisate           | 63.3                 | 30.3   | 0.2                          |
| 15         | (Z)-Hex-3-enyl benzoate  | 64.3                 | 37.1   | 0.2                          |

other hand have reported that ethyl benzoate and linalool are significantly more abundant than methyl benzoate in the whole fruit extract. From these data it is suggested that the volatile flavour profile reported by Shiota *et al* is more consistent with that found in 'overripe' fruit. From the present authors' experience, feijoa fruit stored for between 10 and 18 days after natural abscission develop a 'sickly/sweet' odour due to the increased biosynthesis of ethyl benzoate. Linalool is now known to be associated only with the skin oil fraction (Shaw *et al* 1989). Quantitatively, methyl anisate, ethyl and (Z)-hex-3-enyl benzoate and the previously unreported isopropyl benzoate made only a minor contribution to the GC profile as did the aliphatic esters, ethyl and (Z)-hex-3-enyl butanoate and (Z)-hex-3-enyl acetate.

The most striking difference between the headspace volatiles of the intact fruit and those studied here was the presence in the flesh extract of C-6 constituents, including hexan-1-ol, (Z)-hex-3-en-1-ol, (E)-hex-2-enal, (Z)-hex-3-enal and hexanal. This result was not unexpected, since the rapid synthesis of C-6 saturated and unsaturated alcohols and aldehydes from enzyme-mediated catabolism of linolenic and linoleic acids in disrupted fruit tissue is well known (eg Eriksson 1979). It is likely that these constituents, together with (Z)-hex-3-enyl acetate, are mainly responsible for the green flavour notes (Heath 1978) found in the volatile flavour of feijoa flesh.

## REFERENCES

- Bollard F G 1981 Prospects for horticulture: a research viewpoint. DSIR Discussion Paper, Publication No ISSN 0110-5221. Department of Scientific and Industrial Research, Wellington.

- Eriksson C E 1979 Review of biosynthesis of volatiles in fruits and vegetables. In: *Progress in Flavour Research*, eds Land D G & Nursten H E. Applied Science, London, p 159.
- Hardy P J, Michael B J 1970 Volatile components of feijoa fruits. *Phytochemistry* **9** 1355.
- Heath H B 1978 *Flavour Technology: Profiles, Products, Applications*. AVI Publishing, Westport, CT.
- Shaw G J, Ellingham P E, Birch E J 1983 Volatile constituents of feijoa—headspace analysis of intact fruit. *J Sci Food Agric* **34** 743.
- Shaw G J, Allen J M, Visser F R 1985 Volatile flavour constituents of Babaco fruit (*Carica pentagona*, Heilbora). *J Agric Food Chem* **33** 795.
- Shaw G J, Allen J M, Yates M K 1989 Volatile constituents in the skin oil from *Feijoa sellowiana*. *Phytochemistry* **28** 1529.
- Shiota H, Minami T, Tsuneya T 1980. The aroma constituents of strawberry-guava (*Psidium cattelainum* sabine), yellow guava (*Psidium cattelianum* Sabine var. *lucidun* Hort) and Ananus-guava (*Feijoa sellowiana* Berg). *Koryo* **128** 25.



## **Anchored Ratio Scale Values in the Assessment of Perceived Sweetness, Bitterness and Chocolate Flavour Intensity in Chocolate Confetti and Flakes**

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### **ABSTRACT**

*The sensory profile of a particular food product depends on the context of sensations of the products with which that particular product is compared. This implies that the profile obtained is only valid for the period of time during which the physicochemical composition of each of the investigated products remains constant. This disadvantage can be overcome by assessing the perceived intensities of the products within the context of clearly defined physicochemical stimuli which are known to elicit a constant sensory impression over a long period of time. In the present study the perceived sweetness of 16 brands of chocolate confetti and flakes was anchored and assessed within the context of different sucrose solutions by 31 subjects. The bitterness and chocolate flavour intensities were anchored and assessed within the contexts elicited by different solutions of a mixture of quinine sulphate and caffeine, and cocoa powder/sucrose solutions, respectively. The subjective intensities were assessed on a validated ratio scale by making use of a functional measurement approach. Any changes in the sweetness, bitterness or chocolate flavour intensities of these products are now detectable over a long period of time.*

**Key words:** Sensory analysis, functional measurement, rating scale, sweetness, bitterness, chocolate flavour, cocoa products.

### **INTRODUCTION**

When assessing the sensory profile of a particular food product, the sensory perception of that product is usually compared with the perception of other brands

or product varieties within the same product category. The perceived intensities of the relevant perceptual attributes of the food products are usually obtained by using a particular type of rating scale or magnitude estimation (eg Amerine *et al* 1965; Moskowitz 1983; O'Mahony 1986).

Although this method of assessment can yield accurate results, the approach has two main disadvantages. In the first place there is a disadvantage arising from the context of the stimuli used. Altering the composition of one product changes the context of perceived intensities (McBride 1985) and leads to a different sensory profile for each of the products originally involved. That is to say that the sensory profile of a particular product is only valid as long as no changes have occurred in the products screened. As it is unlikely that a whole series of food products will remain unchanged over a long period of time, any sensory profile obtained is valid for only a limited time period.

The second disadvantage is the result of the use of a particular type of rating scale. The scaling methods normally used provide no checks on the psychometric properties of the responses obtained. This implies that the relationship between observed responses and subjective intensities is unknown. From the statistics that are usually applied, it can be inferred that it is a general and implicit assumption that the responses are linear with subjective intensity. However, it appears that this assumption is not generally valid (Birnbaum 1982).

This paper illustrates a methodology to resolve the two problems referred to above. The context of the stimuli was controlled by presenting the samples within the context of well defined stimuli, of which the physicochemical composition and its sensory perception are known to remain constant over a long period of time. In order to obtain a validated interval scale of perceived taste intensity, a functional measurement approach in combination with a dual stimulus procedure was used (Anderson 1981, 1982; Birnbaum 1982). This scaling method provides an internal check on whether or not the observed responses are linear with perceived intensity. This check can be illustrated by the following example.

Suppose that there are three stimuli, A, B and C, having perceived intensities  $I_a$ ,  $I_b$  and  $I_c$ , respectively. Subjects are presented with the pairs AB, AC and BC, and requested to rate the differences in perceived intensity between the two stimuli of each pair. If the subjects' responses are linear with differences in perceived intensity (ie the responses are measured on an interval scale), then the response on pair AC must be equal to the sum of the responses to pairs AB and BC. If the subjects' responses are not linear with differences in perceived intensity, then the response on pair AC is not equal to the sum of the responses to pairs AB and BC. This method of reasoning can be extended to more than three stimuli. In the case of more than three stimuli, the responses are plotted as a function of the second stimulus of a pair with a separate curve for each first stimulus of a pair. Such a figure is called a factorial plot. The linearity of the responses can be evaluated by inspecting whether or not the curves in such a factorial plot run parallel to each other. Parallelism in a factorial plot can be tested statistically by ANOVA. If the interaction between the first and second stimulus is not statistically significant, then it can be concluded that the curves run parallel.

This scaling method has recently been applied in taste psychophysics (De Graaf

*et al* 1987; De Graaf and Frijters 1988). If it is assumed that water has zero perceived taste intensity, this scaling method yields interval scales of perceived taste intensity with an absolute and meaningful zero point. An interval scale with a absolute zero point is a ratio scale.

Applying the functional measurement approach in combination with a dual stimulus procedure to a series of food products soon leads to an unacceptable number of pairs to be judged as the number of products increases. For example, when applying this method with 10 stimuli, 100 pairs of stimuli (ie a total of 200 items) have to be judged. In this study, the functional measurement approach was applied with a limited number of stimuli. This was the first part of the study. The same stimuli used in this functional measurement procedure were also used in the second part of this study in which a single stimulus procedure was employed. By comparing the results of the first and second parts of the study, it could be established that the responses in the second part were linear with perceived taste intensity.

The entire study resulted in validated ratio scale values for the perceived sweetness, bitterness and chocolate flavour intensity for commercial brands of chocolate confetti and flakes. Any changes in the subjective intensities of these products can be measured over a long period of time. The perceived intensities obtained can also be compared with similar intensities obtained from products of other product categories.

## METHODS AND MATERIALS

The entire study consisted of two separate investigations, each comprising three similarly designed experiments, one for sweetness, one for bitterness and one for assessing the chocolate flavour intensity. In the first investigation the functional measurement approach in combination with a dual stimulus procedure was applied. This investigation was carried out to obtain validated ratio scale values for the perceived intensity of stimuli which were accurately defined from a physicochemical point of view. In the second investigation, the different brands of chocolate confetti and flakes were judged within the context of the stimuli that had been presented in the first investigation.

### Subjects

The subjects were 31 paid volunteers, 19 women and 12 men, varying in age between 19 and 25 years. All of them were students from the Agricultural University. Subjects were selected out of a group of 52 volunteers on the basis of the internal consistency of responses in a preliminary sensory experiment with chocolate flakes and confetti.

### Stimuli

The stimuli used in the first investigation for sweetness intensity were solutions of commercial sucrose (CSM-Breda) in tap water. The concentrations were 0.00 (water), 0.125, 0.25, 0.50 and 1.00 M sucrose. The solutions presented in the second

investigation for sweetness were 0.00 (water), 0.125, 0.177, 0.25, 0.354, 0.500, 0.707, 1.00 and 1.414 M sucrose.

The bitterness stimuli used in the first investigation were solutions of mixtures of quinine sulphate (Sigma Q-1250) and caffeine (Sigma C-0750) in tap water. A mixture of two bitter substances was used in order to reduce the chance of bias due to possible differences in sensitivity to one of the substances. The concentrations were 0.00 (water), 1.77, 3.54, 7.08 and 14.16 mM of an equiratio mixture of quinine sulphate and caffeine (QuiCaf). An equiratio mixture is defined as a series of mixtures with varying concentrations but with a constant ratio (0.0113/0.9887 in this QuiCaf) between its constituent components (Frijters and Oude Ophuis 1983). The measure of concentration for an equiratio mixture is the total concentration of the mixture. The concentrations used in the second investigation for bitterness were 0.00 (water), 1.77, 2.50, 3.54, 5.06, 7.08, 9.11, 14.16 and 17.20 mM of QuiCaf.

The stimuli used to obtain scale values for perceived chocolate flavour intensity were mixtures of two commercially available cocoa powders and commercial sucrose (CSM-Breda) dissolved in tap water. The alkylated cocoa powders (type D-11-A, cacaofabriek de Zaan BV, Koog aan de Zaan and a similar type from Gerkens, Amsterdam) containing 100–120 g kg<sup>-1</sup> fat and were mixed on an equal weight basis. This mixture was in turn mixed on an equal weight basis with sucrose (CocoSucr). The concentrations of CocoSucr used in the first investigation were 0.00 (water), 25, 50, 100, 200 and 400 g litre<sup>-1</sup> and in the second investigation 25, 35, 50, 71, 100, 141, 200, 282 and 400 g litre<sup>-1</sup>.

In both the first and second investigations two stimuli were presented to the subjects in order to accommodate them to the ends of the graphical scales. These stimuli were water and 1.50 M sucrose for sweetness, water and 20 mM QuiCaf for bitterness, and water and 500 g litre<sup>-1</sup> CocoSucr for chocolate flavour experiments.

The samples of chocolate confetti consisted of five brands of 'plain' chocolate confetti coded A–E and five brands of 'milk' chocolate confetti coded F–J. The confettis E and J contained cocoabutter replacers and less cocoa powder than the level at which sale as chocolate confetti is permitted by Dutch legislation. They are sold under the name 'Cocoa Fantasy'. Besides the different brands of chocolate confetti, there were six brands of chocolate flakes coded K–P. The codes K, M and P were brands of 'plain' chocolate flakes, the codes L and N were brands of 'milk' chocolate flakes and the code O was a brand of 'Cocoa Fantasy'.

### Experimental design

The five stimuli in the first investigation for assessing validated scale values of sweetness were presented pairwise to the subjects according to a five-by-five factorial judgement design' (De Graaf *et al* 1987). Each of the five stimuli served five times as first stimulus of a pair and five times as second stimulus of a pair. In the first investigation for sweetness, subjects were presented with 25 pairs. A similar design was used for the first investigation for bitterness. Subjects were presented with the 25 pairs of the five-by-five factorial judgement design. The six stimuli in the first investigation for the chocolate flavour were presented according to a six-by-six factorial judgement design, yielding 36 pairs of stimuli. Each stimulus was presented six times as first stimulus and six times as second stimulus.

In the second investigation, subjects were presented with 52 single stimuli for sweetness, in two series of 26 stimuli. Each series comprised the 16 brands of confetti or flakes and the nine sucrose stimuli (water included). As a control, 0.25 M sucrose was presented twice within each series. For bitterness, subjects were also presented with 52 stimuli in a manner similar to that used for sweetness. Each of the two series consisted of the 16 brands of chocolate confetti and the nine stimuli of the series of QuiCaf. As a control 5.06 mM QuiCaf was presented twice within each series. For chocolate flavour, subjects were presented also with 52 stimuli, two series of 26, where each series consisted of 16 brands of confetti and flakes and ten CocoSucr solutions.

## Procedure

As the experimental procedures were similar for sweetness, bitterness and chocolate flavour, only that for sweetness is explained in full. Specific departures from the general procedure are referred to where appropriate.

### *First investigation*

The subjects were instructed to judge the direction and magnitude of the difference between the perceived sweetness intensities of the two stimuli of each pair. The responses were marked by a slash on a visual analogue scale of 250 mm (De Graaf *et al* 1987), of which the middle was defined as 'the first and second stimulus have equal perceived sweetness intensity'. Subjects were to mark a slash on the right-hand side of the scale if the second stimulus of the pair was perceived as being sweeter and on the left-hand side if the first stimulus of the pair was perceived as being sweeter. The left and right ends of the scale were anchored with the term 'maximum difference', defined as the difference in perceived intensity of the two stimuli presented at the beginning of the experiment.

The pairs of stimuli were presented in a different random order for each subject. The time interval between the tasting of the stimuli was 50 s, both within and between pairs. The stimuli were presented at room temperature in hard plastic polystyrene medicine cups containing about 10 ml solution. When judging the cocoa powder/sucrose solutions, subjects were requested to stir the suspension with a plastic spoon in order to ensure that the cocoa powder particles were homogeneously distributed throughout the suspension.

### *Second investigation*

The subjects were instructed to judge the sweetness intensity of each single stimulus. The responses were marked on a visual analogue scale of 15 cm. The left-hand side of this scale was anchored with the term 'first stimulus' and the right-hand side with the term 'second stimulus'. The terms first and second stimulus referred to the perceived taste intensities of the two stimuli presented at the beginning of each experiment. Subjects were encouraged to use the same units on the scale as they did with the first investigation.

The stimuli were presented in two series of 26 stimuli in a different random sequence for each series and each subject. The time interval between two stimuli was 1 min. Further details of the procedure were the same as for the first investigation.

## Data analyses

Analysis was carried out whether or not, in the first investigation, the response output function, ie the relationship between perceived differences and observed responses, was linear. This was done by examining a 'factorial plot' of the responses, ie a graph in which the responses were plotted as a function of the concentration of the second stimulus, with a separate curve for each concentration level of the first stimulus. If the lines in this factorial plot ran parallel, the response output function was linear. Parallelism in a factorial plot is tested statistically by using ANOVA. Absence of a significant first/second interaction indicates parallelism (Anderson 1981, 1982).

If the response output function is linear, then the row and column means of the response matrix are a linear function of the perceived intensities of these stimuli (Anderson 1981, 1982). Each response matrix yields two sets of marginal means, one for the stimuli tasted as first stimulus of each pair and one for the stimulus (in this case the same one) tasted as second stimulus of each pair. The marginal means of water were set equal to zero, and the scale values of the other stimuli were obtained by calculating the absolute distance to the scale value of water (De Graaf *et al* 1987). The final scale for each stimulus in the first investigation was obtained by averaging its row and column scale value.

If the response function in the second investigation was also linear, then the scale values of the stimuli tasted in the second investigation may have differed by only a multiplicative constant from the scale values of the stimuli tasted in the first investigation. If that turned out to be the case, then the scale values of all stimuli were obtained.

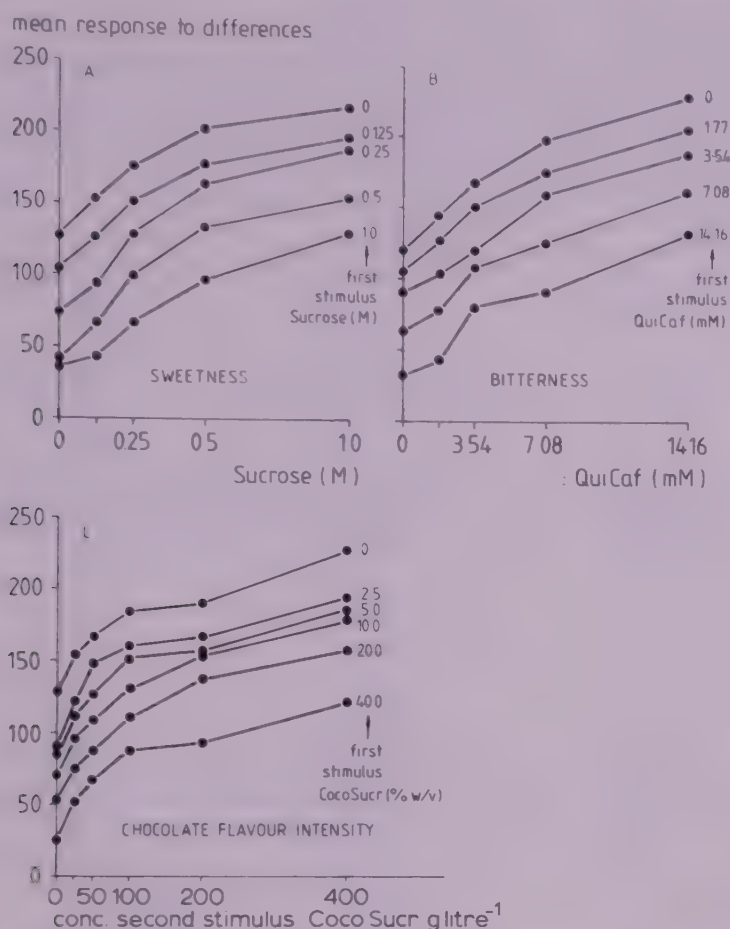
## RESULTS AND DISCUSSION

### The first investigation: functional measurement

In Fig 1 the factorial plots of the responses of the first investigation are shown for sweetness, bitterness and chocolate flavour intensity. Visual inspection suggests that the lines run parallel in each of the three cases. This is confirmed by ANOVA, showing that the first/second interaction is not significant (sweetness:  $F(16, 480) = 1.52$ ,  $P > 0.05$ ; bitterness:  $F(16, 480) = 1.10$ ,  $P > 0.05$ ; chocolate flavour:  $F(25, 750) = 1.48$ ,  $P > 0.05$ ). The parallelism in the factorial plots and the absence of a statistically significant first/second interaction indicates that the response output function in the first investigation was linear.

### Comparison between the first and second investigations

Figure 2 demonstrates the relationships between the scale values of the stimuli tasted in the second investigation and the scale values of the same stimuli tasted in the first investigation. The straight lines through the points in the figure were obtained by orthogonal regression through the origin (Snedecor and Cochran 1976; Hampton 1983). From this figure it is apparent that the scale values of the second investigation differ only by a multiplicative constant from the scale values in the first investigation. Therefore it can be concluded that the response output functions of



**Fig 1.** Mean response to the difference in perceived sweetness (A), bitterness (B) and chocolate flavour intensity (C) between the first and second stimulus of each pair plotted as a function of the concentration of the second stimulus with a separate curve for each concentration of the first stimulus. QuiCaf represents a quinine sulphate-caffeine 0.0113:0.9887 mixture; CocoSucr stands for a 50:50 mixture of cocoa powder and sucrose.

the second investigation are linear, which means that the responses obtained are linear with perceived taste intensity. When it is assumed that water has zero sweetness, bitterness and chocolate taste intensity, these scale values represent validated ratio scale values of perceived taste intensity.

The results in Fig 2 show that the responses obtained with a rating scale can be linear with perceived intensity if sufficient precautions are taken. This result is in agreement with the results and conclusions of other investigators (Birnbbaum 1982; McBride 1983).

### Perceived intensities of the chocolate products

After establishing that the response output functions of the second investigation are linear, the responses for the other stimuli can be calculated as well. Tables 1, 2 and 3 give the ratio scale values for perceived sweetness, bitterness and chocolate flavour intensity, respectively.

Table 1 shows that the sweetnesses of the different chocolate confettis and chocolate flakes vary between 62 and 96 units. Within this range, the different plain brands vary between 62 and 76 units and the different milk brands vary between 84

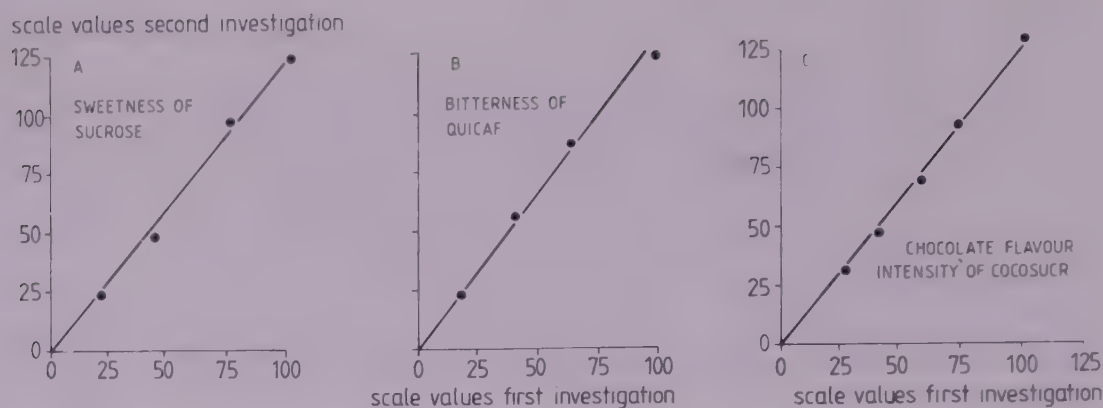


Fig 2. Relationship between scale values of first and second investigation. (QuiCaf = quinine sulphate, caffeine 0.0113/0.9887; CocoSucr = cocoa powder/sucrose 50/50).

TABLE 1

Ratio scale values and their 95% confidence interval for the perceived sweetness intensity of sucrose solutions, ten different brands of chocolate confetti and six different brands of chocolate flakes (second investigation)

| <i>Stimulus</i>               | <i>Code</i> | <i>Perceived sweetness</i> | <i>95% confidence interval</i> |
|-------------------------------|-------------|----------------------------|--------------------------------|
| <b>Sucrose</b>                |             |                            |                                |
| 0.125 M                       |             | 26                         | 21-31                          |
| 0.177 M                       |             | 38                         | 32-44                          |
| 0.250 M                       |             | 52                         | 45-59                          |
| 0.354 M                       |             | 75                         | 67-83                          |
| 0.500 M                       |             | 93                         | 85-103                         |
| 0.707 M                       |             | 107                        | 102-112                        |
| 1.000 M                       |             | 120                        | 114-126                        |
| 1.414 M                       |             | 130                        | 123-137                        |
| Chocolate confetti, plain     | A           | 63                         | 55-71                          |
| Chocolate confetti, plain     | B           | 66                         | 59-73                          |
| Chocolate confetti, plain     | C           | 62                         | 54-70                          |
| Chocolate confetti, plain     | D           | 67                         | 59-75                          |
| Cocoa Fantasy confetti, plain | E           | 72                         | 65-79                          |
| Chocolate confetti, milk      | F           | 93                         | 87-99                          |
| Chocolate confetti, milk      | G           | 89                         | 82-96                          |
| Chocolate confetti, milk      | H           | 87                         | 79-95                          |
| Chocolate confetti, milk      | I           | 85                         | 78-92                          |
| Cocoa Fantasy confetti, milk  | J           | 84                         | 77-91                          |
| Chocolate flakes, plain       | K           | 68                         | 59-76                          |
| Chocolate flakes, milk        | L           | 92                         | 85-99                          |
| Chocolate flakes, plain       | M           | 71                         | 64-78                          |
| Chocolate flakes, milk        | N           | 96                         | 89-103                         |
| Cocoa Fantasy flakes          | O           | 86                         | 79-93                          |
| Chocolate flakes, plain       | P           | 76                         | 68-84                          |

TABLE 2

Ratio scale values and 95% confidence interval for the perceived bitterness intensity of quinine sulphate/caffeine 0.013/0.9887 (QuiCaf), ten different brands of chocolate confetti and six brands of chocolate flakes (for explanation of codes, see Table 1)

| <i>Stimulus<br/>(code)</i> | <i>Perceived<br/>bitterness</i> | <i>95% confidence<br/>interval</i> |
|----------------------------|---------------------------------|------------------------------------|
| QuiCaf (mM)                |                                 |                                    |
| 1.77                       | 25                              | 19–30                              |
| 2.50                       | 43                              | 37–50                              |
| 3.54                       | 56                              | 49–62                              |
| 5.06                       | 75                              | 66–83                              |
| 7.08                       | 86                              | 79–93                              |
| 9.11                       | 108                             | 100–114                            |
| 14.16                      | 119                             | 114–126                            |
| 17.20                      | 120                             | 117–127                            |
| A                          | 50                              | 41–59                              |
| B                          | 47                              | 38–56                              |
| C                          | 42                              | 33–51                              |
| D                          | 47                              | 39–55                              |
| E                          | 22                              | 15–29                              |
| F                          | 27                              | 19–35                              |
| G                          | 30                              | 20–40                              |
| H                          | 24                              | 17–31                              |
| I                          | 23                              | 17–29                              |
| J                          | 13                              | 8–18                               |
| K                          | 49                              | 39–57                              |
| L                          | 26                              | 19–33                              |
| M                          | 36                              | 28–44                              |
| N                          | 16                              | 10–22                              |
| O                          | 19                              | 13–25                              |
| P                          | 41                              | 33–49                              |

and 96. With regard to perceived sweetness intensity it appears that there are two distinct groups, the plain brands and the milk brands. Within the two groups there are no great differences in sweetness intensity. The milk group is substantially sweeter than the plain group.

Table 2 shows the bitterness intensity for the different QuiCaf concentrations and the different brands of chocolate confetti and flakes. The bitterness varies between 13 units for the Cocoa Fantasy flakes (brand J) and 50 units for a brand of plain chocolate confetti (brand A). The plain brands have a bitterness intensity between 36 and 50 units and the milk brands a bitterness intensity between 16 and 30 units. The Cocoa Fantasy varieties have a bitterness intensity of about 20 units or less.

Table 3 shows that the differences in perceived chocolate flavour intensity between the different brands are greater than the differences in perceived sweetness or bitterness. Some plain brands (A, B, D, K, P) have a chocolate flavour intensity of

TABLE 3

Ratio scale values and 95% confidence intervals of perceived chocolate flavour intensity of cocoa powder/sucrose 50/50 (CocoSucr), ten different brands of chocolate confetti and six different brands of chocolate flakes (for explanation of codes, see Table 1)

| <i>Stimulus (code)</i>            | <i>Perceived chocolate flavour</i> | <i>95% confidence interval</i> |
|-----------------------------------|------------------------------------|--------------------------------|
| CocoSucr (g litre <sup>-1</sup> ) |                                    |                                |
| 25                                | 32                                 | 28–38                          |
| 35                                | 41                                 | 36–45                          |
| 50                                | 48                                 | 42–53                          |
| 71                                | 63                                 | 58–69                          |
| 100                               | 71                                 | 67–76                          |
| 200                               | 96                                 | 90–101                         |
| 282                               | 106                                | 106–111                        |
| 400                               | 129                                | 124–133                        |
| A                                 | 76                                 | 67–85                          |
| B                                 | 77                                 | 69–85                          |
| C                                 | 60                                 | 52–68                          |
| D                                 | 79                                 | 70–88                          |
| E                                 | 32                                 | 26–38                          |
| F                                 | 42                                 | 35–49                          |
| G                                 | 44                                 | 37–51                          |
| H                                 | 37                                 | 30–44                          |
| I                                 | 37                                 | 30–44                          |
| J                                 | 28                                 | 23–33                          |
| K                                 | 78                                 | 69–87                          |
| L                                 | 39                                 | 33–45                          |
| M                                 | 57                                 | 48–66                          |
| N                                 | 33                                 | 26–40                          |
| O                                 | 42                                 | 35–49                          |
| P                                 | 73                                 | 64–82                          |

70–80 units. Other plain brands (C, M) elicit a much lower perceived chocolate intensity of about 60 units. The perceived chocolate intensity of the milk and Cocoa Fantasy varieties varies between 28 and 44 units.

### Intensities of chocolate products expressed in physicochemical equivalents

Tables 1, 2 and 3 also show the sweetness, bitterness and chocolate flavour intensity of the stimuli which provided the context wherein the brands of confetti and flakes were judged. These data facilitate expression of the perceived sweetness, bitterness and chocolate flavour of the confettis and flakes at the concentrations of these stimuli that elicit an equivalent sensory intensity. The procedure for arriving at these concentrations is illustrated in Fig 3 which shows the psychophysical function of sucrose and the sweetness of brand K as determined from the data in Table 1. According to Table 1, brand K elicits a sweetness intensity of 68 units. From Fig 3 it

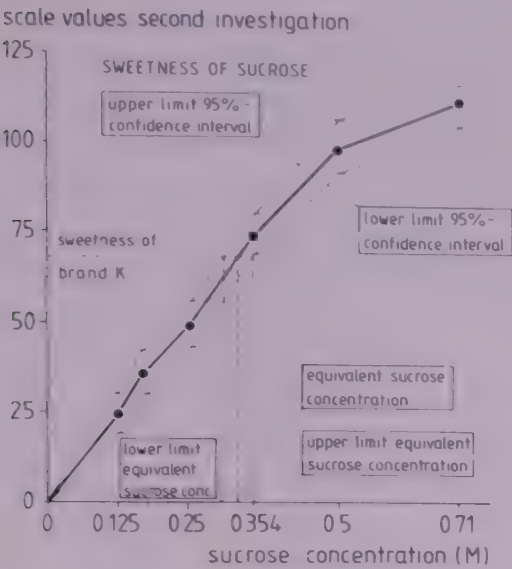


Fig 3. Procedure for determining the sucrose concentration and its 95% confidence interval, which elicits the same sweetness intensity as a brand of plain chocolate flakes (brand K).

TABLE 4  
Concentrations of sucrose (M) and 95% confidence interval which elicit a similar sweetness intensity as the different brands of chocolate confetti and chocolate flakes

| Brand code | Sucrose conc. (M) | 95% confidence interval |
|------------|-------------------|-------------------------|
| A          | 0.33              | 0.26–0.34               |
| B          | 0.31              | 0.28–0.35               |
| C          | 0.29              | 0.26–0.33               |
| D          | 0.32              | 0.29–0.36               |
| E          | 0.34              | 0.31–0.39               |
| F          | 0.51              | 0.44–0.61               |
| G          | 0.47              | 0.41–0.55               |
| H          | 0.46              | 0.39–0.53               |
| I          | 0.44              | 0.38–0.50               |
| J          | 0.43              | 0.36–0.49               |
| K          | 0.33              | 0.29–0.36               |
| L          | 0.49              | 0.43–0.59               |
| M          | 0.34              | 0.30–0.39               |
| N          | 0.55              | 0.46–0.64               |
| O          | 0.44              | 0.38–0.51               |
| P          | 0.37              | 0.33–0.43               |

can be determined by graphical estimation that 0.33 M sucrose will elicit a similar sweetness intensity. Similar concentrations for the sweetness, bitterness and chocolate flavour intensity of the other brands were determined in a similar way. The 95% confidence intervals of these concentrations were determined by similar graphical estimation through the 95% upper and lower confidence limits of the intensities of sucrose, QuiCaf and CocoSucr. This procedure is also illustrated in Fig 3.

Tables 4, 5 and 6 show the concentrations of the stimuli that elicit sweetness,

**TABLE 5**  
Concentrations of quinine sulphate/caffeine 0.113/0.9887 (QuiCaf) and 95 % confidence intervals which elicit a similar bitterness intensity as the different brands of chocolate confetti and chocolate flakes

| <i>Brand code</i> | <i>QuiCaf (mM)</i> | <i>95 % confidence interval</i> |
|-------------------|--------------------|---------------------------------|
| A                 | 3.10               | 2.50–3.65                       |
| B                 | 2.83               | 2.37–3.38                       |
| C                 | 2.48               | 2.21–3.01                       |
| D                 | 2.83               | 1.55–2.10                       |
| E                 | 1.57               | 1.28–1.92                       |
| F                 | 1.81               | 1.55–2.10                       |
| G                 | 1.99               | 1.77–2.28                       |
| H                 | 1.70               | 1.33–1.99                       |
| I                 | 1.66               | 1.32–1.97                       |
| J                 | 0.91               | 0.73–1.19                       |
| K                 | 3.05               | 2.48–3.48                       |
| L                 | 1.80               | 1.50–2.08                       |
| M                 | 2.23               | 1.99–2.54                       |
| N                 | 1.15               | 0.93–1.48                       |
| O                 | 1.39               | 1.13–1.80                       |
| P                 | 2.43               | 2.17–2.88                       |

**TABLE 6**  
Concentrations of cocoa powder/sucrose 50/50 (CocoSucr) and 95 % confidence intervals which elicit a similar chocolate flavour intensity as the different brands of confetti and flakes

| <i>Brand code</i> | <i>CocoSucr g litre<sup>-1</sup></i> | <i>95 % confidence interval</i> |
|-------------------|--------------------------------------|---------------------------------|
| A                 | 116                                  | 100–141                         |
| B                 | 120                                  | 101–146                         |
| C                 | 66                                   | 60–80                           |
| D                 | 130                                  | 110–153                         |
| E                 | 25                                   | 21–31                           |
| F                 | 39                                   | 31–50                           |
| G                 | 41                                   | 33–53                           |
| H                 | 30                                   | 25–38                           |
| I                 | 30                                   | 25–38                           |
| J                 | 23                                   | 19–28                           |
| K                 | 128                                  | 109–151                         |
| L                 | 33                                   | 28–43                           |
| M                 | 59                                   | 54–70                           |
| N                 | 26                                   | 23–33                           |
| O                 | 38                                   | 31–52                           |
| P                 | 108                                  | 88–128                          |

bitterness and chocolate flavour intensities similar to those of the different confettis and flakes. The concentrations stated in these tables depend on three parameters. Obviously, the first parameter is the perceived intensity of the brand itself. The second parameter is the shape of the psychophysical function of the stimulus by which the sensory continuum was manipulated. As the psychophysical functions are not linear, the concentrations in Tables 4, 5 and 6 are non-linearly related to the ratio scale values of Tables 1, 2 and 3. The third parameter is the confidence interval of the scale values in Tables 1, 2 and 3. The concentrations in Tables 4, 5 and 6 enable a quick and accurate sensory screening of different production batches. The sensory intensity of sucrose, QuiCaf and CocoSucr is expected to remain constant over a long period of time. This implies that changes in sweetness, bitterness and chocolate flavour intensity remain measurable over the same long time period.

## CONCLUSION

The main conclusion to be drawn from the results is that the perceived sweetness, bitterness and chocolate flavour intensities of chocolate confetti and flakes have been assessed on a validated ratio scale of subjective intensity. These intensities were judged within the context of subjective intensities elicited by stimuli with a well known and reproducible physicochemical composition. This implies that the subjective intensities of the confettis and flakes are anchored within this context, and enables the measurement of any changes in the sensory intensities of any particular brand over a long period of time, independent of changes in other brands.

## REFERENCES

- Amerine M A, Pangborn R M, Roessler E B 1965 *Principles of Sensory Evaluation of Food*. Academic Press, New York.
- Anderson N H 1981 *Foundations of Information Integration Theory*. Academic Press, New York.
- Anderson N H 1982 *Methods of Information Integration Theory*. Academic Press, New York.
- Birnbaum M H 1982 Controversies in psychological measurement. In: *Social Attitudes and Psychophysical Measurement*, ed Wegener B J. Erlbaum, NJ, pp 401–486.
- De Graaf C, Frijters J E R 1988 Assessment of the taste interaction between two qualitatively similar taste substances: a comparison between comparison rules. *J Exp Psychol: Hum Percept Perform* **14** 526–538.
- De Graaf C, Frijters J E R, Van Trijp H C M 1987 Taste interaction between glucose and fructose assessed by functional measurement. *Percept Psychophys* **41** 383–392.
- Frijters J E R, Oude Ophuis P A M 1983 The construction and prediction of psychophysical power functions of equiratio sugar mixtures. *Percept* **12** 753–767.
- Hampton L A 1983 A note on describing the linear relationship between a pair of correlated dependent variables. *Bull Br Psychol Soc* **36** 408–410.
- McBride R L 1983 A JND/category-scale convergence in taste. *Percept Psychophys* **34** 77–83.
- McBride R L 1985 Stimulus range influences intensity and hedonic ratings of flavor. *Appetite* **6** 103–114.
- Moskowitz H R 1983 *Product Testing and Sensory Evaluation of Foods: Marketing and R and D Approaches*. Food and Nutrition Press, Westport, CT.
- O'Mahony M 1986 *Sensory Evaluation of Food*. Marcel Dekker, New York.
- Snedecor G W, Cochran W G 1976 *Statistical Methods* (6th edn). Iowa University Press, Ames, Iowa.



## Quantitation of Soya Protein by Enzyme Linked Immunosorbent Assay of its Characteristic Peptide

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### ABSTRACT

*An enzyme linked immunosorbent assay (ELISA) is described for the quantitative measurement of soya proteins in processed food products. The peptide fragment bearing an antigenicity against anti-glycinin (soya bean 11S globulin) antibody was purified from the trypsin digests of autoclaved glycinin and used as an indicator antigen for soya proteins. ELISA samples were prepared also by a combination of autoclaving and tryptic digestion. Quantitation of soya proteins by ELISA was found to resist interference by other food materials, and the ELISA signal was independent of soya bean cultivar. The ELISA method was evaluated on pork sausages which were prepared in the laboratory to contain 0 to 20.8% soya protein isolate. With commercial soya protein isolate as reference standard, the results showed that the detected amounts were in quantitative agreement with the added amounts of soya protein isolate.*

**Key words:** Soya protein, glycinin, 11S globulin, antigenic peptide, ELISA, sausage, *Glycine max*.

### INTRODUCTION

Soya bean (*Glycine max* L, Merr) is a traditional crop in east and south-east Asian countries. Many soya-bean-based foods have been developed and utilised there throughout past centuries. With developing technology it has become possible to

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incorporate various forms of soya protein products, such as isolate, concentrate, flour, textured, etc, into processed foods as an ingredient, an extender or as an analogue. The new and improved products thus developed have a wide range of nutritional and functional properties to suit different food systems in many countries. Today soya proteins have become one of the most important protein ingredients in the world's food supply (Wilcke *et al* 1986).

Soya proteins are being applied extensively, particularly in meat products as a partial replacement for animal proteins (Rakosky 1975). However, the addition of soya proteins to meat products for human consumption has been legally restricted in many countries. One of the reasons for this restriction arises from the lack of suitable and sensitive methods for the determination of admixed soya protein in meat products which may be cooked during processing.

To date, many different methods have been suggested for the determination of admixed soya proteins, and several extensive reviews and evaluations have been published (Olsman and Hitchcock 1980; Eldridge 1981; Olsman *et al* 1985; Agater *et al* 1986). These methods of analysis distinguish soya protein from intrinsic meat proteins and other ingredients depending on their differences in histological (Coomaraswamy and Flint 1973; Flint and Meech 1979), physical (Parsons and Lawrie 1972; Guy *et al* 1973; Lee *et al* 1975; Poli *et al* 1979), chemical (Lindqvist *et al* 1975; Bailey 1976; Bailey *et al* 1978; Llewellyn *et al* 1978; Eldridge and Holmes 1979; Armstrong *et al* 1982; Medina and Phillips 1982), and serological (Kamm 1970; Koh 1978; Hitchcock *et al* 1981; Griffiths *et al* 1984; Ravestein and Driedonks 1986; Medina 1988) properties.

Denaturation of protein by heating, particularly under high pressures, renders its chemical and physical properties hardly distinguishable by many of these methods and consequently diminishes the sensitivity and accuracy of determination. By contrast, protein denaturation rarely affects its primary structure. Therefore, protein species in admixture can be identified through their characterising peptides. Peptides characteristic of soya proteins have been analysed in the trypsin hydrolysates by ion exchange chromatography (Bailey 1976; Bailey *et al* 1978; Llewellyn *et al* 1978) and by sequential application of TLC and HPLC (Medina and Phillips 1982). However, these chromatographic techniques suffer from interference by coexisting non-soya components.

Enzyme linked immunosorbent assay (ELISA) has been considered as one of the best methods in terms of its high specificity and sensitivity of detection for the target proteins and peptides, and large throughput of samples (Eldridge and Holmes 1979; Olsman *et al* 1985; Medina 1988). An ELISA kit developed for soya protein determination is commercially available (Rittenburg *et al* 1987). Soya protein determination by ELISA is also liable to be affected by protein denaturation. The ELISA procedure which uses antiserum raised against native soya protein was shown to be unsuitable for sterilised products (Griffiths *et al* 1984). The other ELISA procedure involving extraction of soya proteins under denaturing conditions and making antibodies against the renatured protein is reported to give poor agreement with added amount of soya protein for some products (Koh 1978; Ravestein and Driedonks 1986).

This paper describes an ELISA procedure for the quantitative detection of the

soya protein in heat-treated food products. The principle is based on the antigenicity of peptide fragments characteristic of soya protein. A characteristic peptide was purified from the tryptic digests of soya protein by monitoring its immunoreactivity against the affinity-purified anti-glycinin antibody. The immunoreactive peptide was utilised as the marker for soya proteins. The specificity and reliability of the assay was assessed for soya proteins in different cultivars and in mixed products.

MATERIALS AND METHODS

Materials

Defatted soya bean meal, unheated soya protein isolate (SPI), and commercial SPI (Fujipro E) were gifts from Fuji Seiyu Co (Osaka). Lean beef, pork and hen's egg were purchased at the local market. Food-grade milk casein and wheat gluten were from commercial sources. Five varieties of soya bean, Raiden, Matsuura, Shiotsurunoko, York and Hill, were provided by Mr Kyuya Harada, National Institute of Agrobiological Resources, Ministry of Agriculture, Forestry and Fisheries, Tsukuba. Trypsin crystallised from porcine pancreas (type IX) was from Sigma Chemical Co SP-Toyopearl 650S, DEAE-Toyopearl 650S and Toyopearl HW-40S were from Tosoh Co (Tokyo), formyl Cellulofine was from Chisso Co (Tokyo), and reverse phase HPLC column Pep-RPC HR 5/5 was from Pharmacia Fine Chemicals AB.

Preparation of pork sausage

Pork sausages were prepared in the laboratory by the basic formulation shown in Table 1. The extender emulsion was prepared by blending commercial SPI, pork fat and water in mass proportions of 1:1:4. Pork ham and pork belly were chopped first with crushed ice. In sausages containing SPI, the chopped meat was mixed with the extender emulsion in proportions to provide SPI concentrations of 0, 104 and 208 g kg<sup>-1</sup> of the total protein in the final products. Pyrophosphate and salt were added and chopping was resumed briefly. Nitrite and flavourings were then added

TABLE 1  
Basic formulation for pork sausage

| <i>Ingredient</i>   | <i>Weight (g)</i> |
|---|-------------------|
| Pork ham (200 g kg <sup>-1</sup> protein, 75 g kg <sup>-1</sup> fat)    | 30                |
| Pork belly (130 g kg <sup>-1</sup> protein, 400 g kg <sup>-1</sup> fat) | 50                |
| Ice   | 20                |
| Sodium pyrophosphate  | 0.7               |
| NaCl  | 1.7               |
| Sodium nitrite  | 0.01              |
| Spice mixture   | 0.4               |
| Flavour mixture   | 0.2               |
| Total   | 103.01            |

and chopping was continued for a short time. The mixtures were stuffed into sausage casings, and the sausages were cooked at 80°C for 20 min. After cooling they were stored at -20°C until analysed.

### Preparation of glycinin

Crude glycinin (11S globulin fraction) was prepared from unheated SPI by the method of Thanh and Shibasaki (1976) and was subsequently purified by the procedure outlined below. The glycinin fraction was dialysed against 0.035 M potassium phosphate containing 0.01 M 2-mercaptoethanol and 0.15 M NaCl at pH 7.8, and the retentate was applied to a DEAE-Toyopearl 650S column. Glycinin was eluted with a linear concentration gradient of NaCl from 0.15 to 0.35 M in the same buffer used for dialysis. The fractions were combined, adjusted to pH 7.6 with NaOH, and brought to 80% saturation with ammonium sulphate (4.25 M). The precipitate formed after standing overnight at 4°C was collected, dispersed in 0.1 M sodium phosphate buffer (pH 7.0) containing 0.4 M NaCl, and dialysed against the dispersing buffer. The concentration of glycinin was calculated using the value of  $E_{280\text{ nm}}^{1\% 1\text{ cm}} = 8.04$  (Koshiyama 1972). Purified glycinin was stored at 4°C.

### Antisera production

Antisera were produced by injecting Japanese White rabbits intramuscularly with a dose of 10 mg of glycinin dissolved in 1.0 ml of phosphate-buffered saline (PBS, 0.15 M NaCl-0.01 M sodium phosphate at pH 7.2) and subsequently emulsified in an equal volume of Freund's complete adjuvant, followed by similar booster injections at the third and fifth weeks after the initial injection. Rabbits were bled at intervals. Antibody titres of the sera were monitored by Ouchtalony double diffusion tests in agar.

### Preparation of affinity-purified antibody

To the rabbit serum (4 ml) was added ammonium sulphate to 40% saturation (1.66 M). The precipitates were collected by centrifugation and dialysed against two changes of PBS. After centrifugation to remove the precipitates formed during dialysis, the retentate containing about 140 mg protein was placed on an affinity column prepared by coupling 55 mg of glycinin to 3 g (wet weight) of formyl-Cellulofine and previously equilibrated with PBS. After washing the column with PBS, the antibodies were eluted with 0.2 M glycine-HCl, pH 2.2. The column effluent was neutralised immediately after elution with 0.4 M potassium phosphate, pH 8.0, dialysed against PBS and then concentrated to a small volume by ultrafiltration. The final preparation containing about 15 mg protein in 3 ml was stored at -80°C until use.

### Sample preparation for ELISA

#### Acetone extraction

Sausage samples (10 g) were homogenised in 100 ml of acidic ethanol (0.1 M HCl in ethyl alcohol, 750 ml litre<sup>-1</sup>) in a Waring blender. The homogenates were centrifuged at  $18\,000 \times g$  for 15 min. The resultant pellets were washed twice with acidic ethanol and resuspended in 100 ml of acetone. The suspension was shaken

gently for 10 min and then centrifuged again. This acetone extraction was repeated twice. The pellets from the final centrifugation were air dried and finely ground.

Soya bean and other bean samples were soaked overnight in 20 vol of the acidic ethanol, followed by similarly homogenising and extracting with acetone. Commercial SPI, milk casein and wheat gluten were subjected to the following tryptic digestion without acetone extraction.

#### *Tryptic digestion*

The acetone-extracted sample or commercial protein product (250 mg protein) was suspended in 5 ml of 0.03 M Tris/0.5 M NaCl/0.01 M  $\text{CaCl}_2$  (pH 8.1). The suspension was autoclaved for 3 h at 120°C. After cooling, the autoclaved suspension was mixed with 250  $\mu\text{l}$  of aqueous trypsin solution (10 mg  $\text{ml}^{-1}$ ), and the mixture was incubated for 24 h at 37°C. The tryptic digest was terminated by immersing the mixture in a boiling water bath, and the mixture was mixed with 8 ml of PBS. This diluted mixture was centrifuged at  $18\,000 \times g$  for 20 min and the supernate was retained. The precipitate was resuspended in 2 ml of 10 g  $\text{litre}^{-1}$  acetic acid and recentrifuged. The combined supernate was diluted to 20 ml with PBS.

Protein contents were estimated by the micro Kjeldahl method using the nitrogen to protein conversion factor of 6.38 and 6.25 for milk casein and other samples, respectively.

#### **Purification of immunoreactive fragments against anti-glycinin antibody**

Glycinin in 0.03 M Tris/0.5 M NaCl/0.01 M  $\text{CaCl}_2$  (pH 8.1) was autoclaved and digested with trypsin under the same conditions as described in the preceding section for the ELISA sample. The tryptic digests were centrifuged and the sediments were washed twice with 10 g  $\text{litre}^{-1}$  acetic acid. Aliquots of the supernate (equivalent to approximately 8 mg N) were applied to an SP-Toyopearl 650S ion exchange column (20 cm  $\times$  2.2 cm id) equilibrated with 0.01 M phosphate buffer (pH 6.4). After elution with the equilibrating buffer at a flow rate of 3  $\text{ml min}^{-1}$ , peptides were eluted with a linear gradient from 0 to 1.0 M NaCl at a gradient rate of 0.125 M per 10 ml. The effluent was analysed for the immunoreactivity to anti-glycinin antibody by ELISA. The immunoreactive fractions were collected and lyophilised.

The lyophilised peptides were dissolved in a small volume of 50 mM ammonium hydrogen carbonate solution and charged on a Toyopearl HW-40S gel filtration column (55 cm  $\times$  1.6 cm id). Immunoreactive peptides from gel filtration were further purified by Pep-RPC HR5/5 reversed phase column chromatography (5.0 cm  $\times$  0.5 cm id), which was equilibrated with 1 g  $\text{litre}^{-1}$  trifluoroacetic acid (TFA). Peptides were eluted with a linear concentration gradient of the second solvent (1 g  $\text{litre}^{-1}$  TFA in 600 g  $\text{litre}^{-1}$  acetonitrile) from 0 to 600 g  $\text{litre}^{-1}$  at a gradient rate of 2 g  $\text{litre}^{-1} \text{ min}^{-1}$ . The flow rate of solvent was kept at 0.4  $\text{ml min}^{-1}$ .

#### **ELISA procedure**

A two-step competitive ELISA procedure (Engvall 1980) was used with the enzyme-labelled antibody and the target antigen attached to a solid phase for the assay of antigenic soya peptides by the sequence shown in Fig 1. A whole tryptic digest of glycinin was used as the target antigen in the purification of immunoreactive

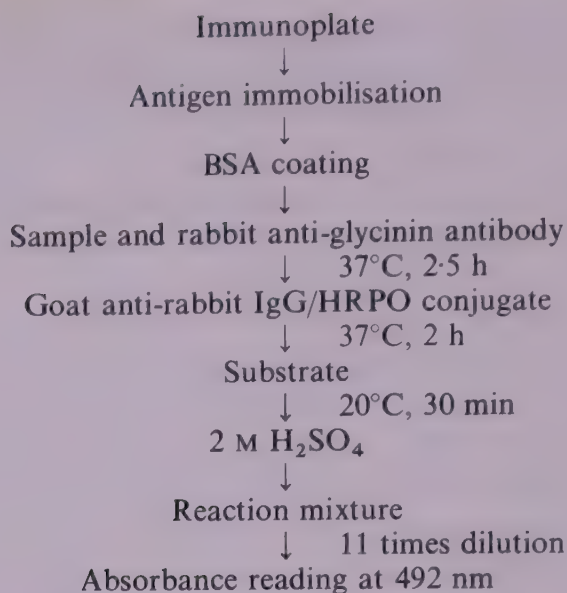


Fig 1. Flow sheet for ELISA procedure. BSA, bovine serum albumin; IgG/HRPO, immunoglobulin G horseradish peroxidase.

peptides, and a purified peptide fraction (S-2-A-6 fraction in Fig 5) in the quantitative analyses of soya protein and in the examination of cross-reactivity of other food materials. Antigen solution (0.1 ml) was added to each well of microtitre plates (Nunc-Immuno Plate I, Nunc Co, Roskilde, Denmark), which were then incubated at 37°C for 2 h. In order to block non-specific protein adsorption sites on the microtitre wells, the antigen-coated wells were emptied by suction, filled with 0.1 ml aliquots each of 10 g litre<sup>-1</sup> bovine serum albumin in PBS (Ravestien and Driedonks 1986) and then incubated for 2 h at 37°C. Each plate was then washed three times with PBS containing 5 g litre<sup>-1</sup> Tween 20 (PBST).

Mixtures of 0.05 ml each of antibody solution and unknown or standard sample solution were added to the antigen-coated wells and the plates were incubated at 37°C for 2.5 h. Each well was washed as described previously and 0.1 ml of the second antibody, peroxidase conjugated affinity-purified goat anti-rabbit IgG (Jackson Immunoresearch Lab, Avondale, PA, USA), in a dilution of 1:5000 in PBST was added. After reincubation for 2 h at 37°C and subsequent washing with PBST, 0.15 ml of the enzyme substrate solution (prepared by dissolving 8 mg *o*-phenylenediamine in 20 ml of 0.1 M citrate/0.2 M phosphate buffer, pH 5.0, containing 4.0 µl of 300 g litre<sup>-1</sup> H<sub>2</sub>O<sub>2</sub>) was placed in each well. The enzyme reaction was stopped after incubation for 30 min at 20°C by the addition of 0.05 ml of 2 M H<sub>2</sub>SO<sub>4</sub> to each well. The absorbance in each well was read at 492 nm after 11-fold dilution with 1.2 M H<sub>2</sub>SO<sub>4</sub>.

The calibration standards were prepared from SPI digest by serial dilution into PBST. Test samples were also serially diluted; tryptic digests of pork sausage twice and other samples four times with PBST. Anti-glycinin antibody was diluted 500-fold with PBST.

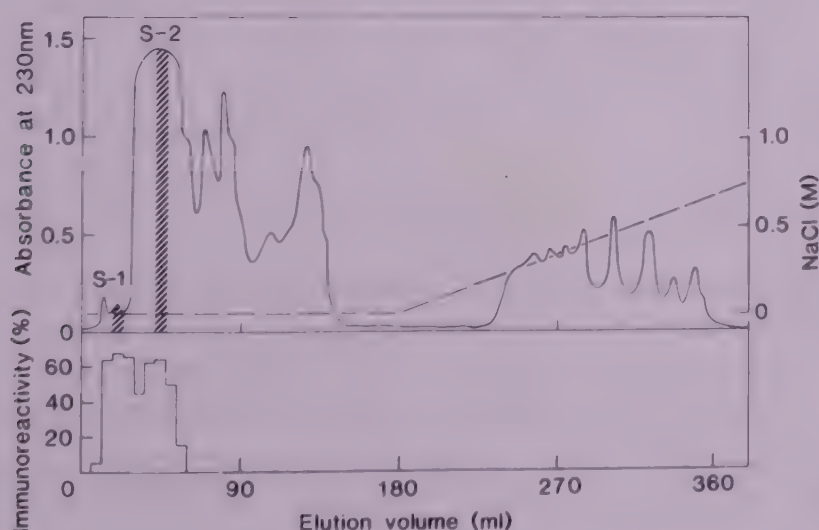
## RESULTS

### Antisera

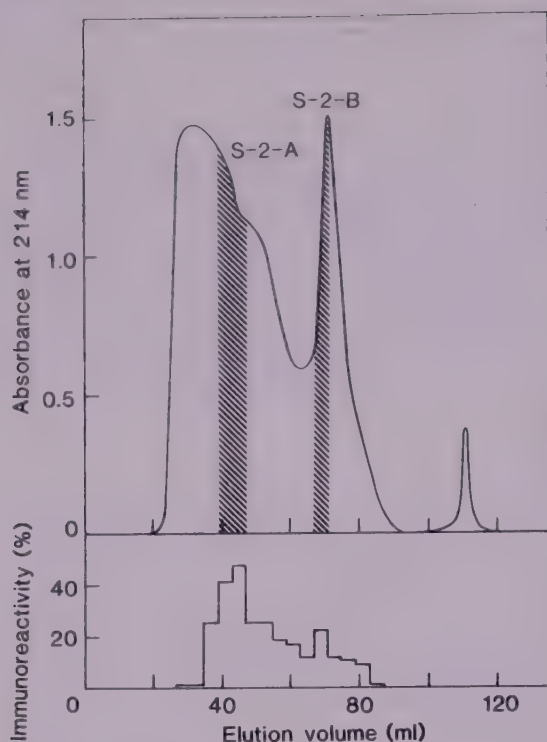
All the rabbits immunised with soya glycinin raised antisera. Anti-glycinin antibodies were purified, by the procedure outlined in 'Materials and methods', from antisera that showed optimal titres. The purified antibodies gave a lower background than the crude antisera in the ELISA test. The high background observed with the crude sera may be due to specific and non-specific binding of antibodies to the coated plastics and probably to other protein/protein interactions as well.

### Purification of immunoreactive peptides

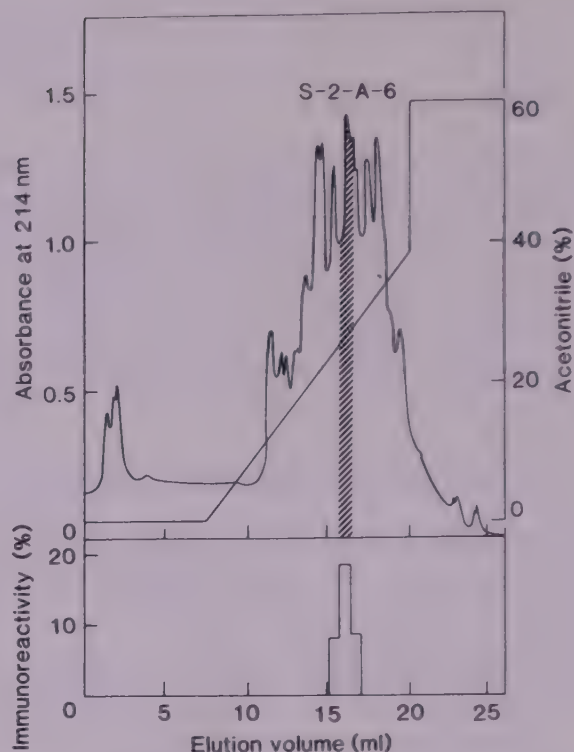
The immunoreactive peptides were purified from the tryptic digests of glycinin by combination chromatography as described in 'Materials and methods'. Immunoreactivity was detected by the ELISA procedure using the tryptic digests of glycinin as the immobilised antigens. Figure 2 shows the elution profile of the tryptic digest from an SP-Toyopearl 650S column. Two peptide peaks, resolved near the void volume of the column and designated S-1 and S-2, showed positive immunoreactivity to the antibodies. These two peak fractions were collected and lyophilised individually. On Toyopearl HW-40S gel filtration, S-1 gave a single immunoreactive peak which was eluted near the void volume of the column (data not shown), whereas S-2 resolved further into two active fractions designated S-2-A and S-2-B in Fig 3. The fraction S-1 gave a high background (data not shown) in ELISA, thereby reducing significantly the sensitivity of detection. The fractions containing the larger peptides in the gel filtration, which were poorly resolved, gave a higher titre in ELISA than the S-2-B peptide fraction. The two fractions were individually pooled and lyophilised. Peptides contained in the fraction S-2-A were resolved further on a Pep-RPC HR 5/5 reversed phase column. Figure 4 shows that



**Fig 2.** Elution profiles of trypsin-digested soya bean glycinin from SP-Toyopearl 650S column. Solid line in the upper frame shows elution pattern monitored at 230 nm, and dotted line NaCl concentration in the gradient elution. Solid line in the bottom frame shows the immunoreactivity of the fractions to anti-rabbit 11S antibody in the ELISA assay.



**Fig 3.** Gel filtration profile of S-2 fraction from Toyopearl HW-40S column. Eluate was monitored at 214 nm (upper frame). The fraction S-2-A (shaded) was collected for further purification.



**Fig 4.** Elution pattern of S-2-A fraction from PepRPC HR 5/5 reverse phase column in FPLC system (Pharmacia Fine Chemicals AB) with gradient of acetonitrile concentration from 0 to 400 g litre<sup>-1</sup> in 10 g litre<sup>-1</sup> trifluoroacetic acid (dotted line). Elution profile was recorded by monitoring absorbance at 214 nm. The fraction denoted as S-2-A-6 (shaded) was collected.

among the resolved fractions only one peak fraction designated S-2-A-6 positively reacted with the antibodies.

The purification procedure described above provided three antigenic fractions, S-1, S-2-A-6 and S-2-B. Among them, fraction S-2-A-6 showed the highest specific reactivity against the affinity-purified antibodies, giving the lowest background readings in the ELISA test. Hence the fraction S-2-A-6 was chosen as the marker for soya protein.

### Calibration curve and limit of detection

The calibration curve for the ELISA determination of soya protein was set up using commercial SPI as reference standard. The target antigen attached to the solid phase was the fraction S-2-A-6 peptide. In Fig 5 are plotted the absorbance readings against the concentrations of the standard SPI on a semi-logarithmic scale. For this curve, the effective range of assay was found to be from 2.4 to 312.5 µg per well and the lower limit for detection of soya bean protein was found to correspond to ~ 0.4 g SPI per 100 g of total protein.

### Response to different soya bean varieties and non-soya food protein

Table 2 compares the reactivity of representative soya bean varieties, Matsuura,

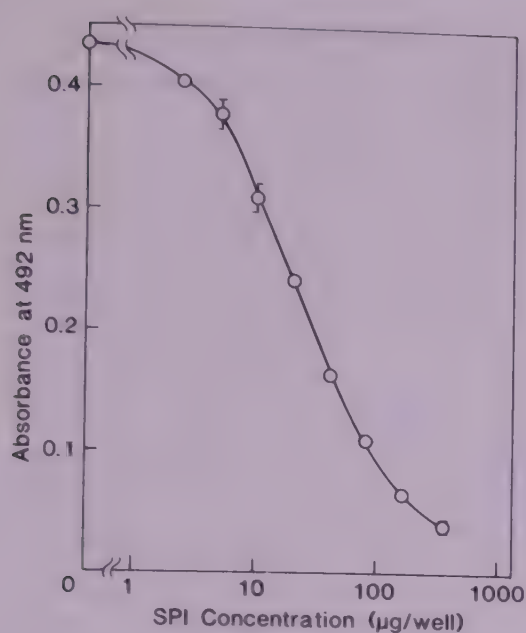


Fig 5. ELISA calibration curve for soya glycinin.

**TABLE 2**  
Response of soya bean proteins of different cultivars to ELISA

| Variety       | Response <sup>a</sup> |
|---------------|-----------------------|
| Matsuura      | 100                   |
| Raiden        | 97                    |
| Shiotsurunoko | 89                    |
| York          | 90                    |
| Hill          | 92                    |

<sup>a</sup> Averages of duplicate determinations are recorded relative to SPI (Fujipro E = 100).

**TABLE 3**  
Response of food materials to ELISA

| Sample     | Response <sup>a</sup> |
|------------|-----------------------|
| SPI        | 100                   |
| Beef       | ND                    |
| Pork       | 0.9                   |
| Egg        | ND                    |
| Gluten     | 1.5                   |
| Casein     | ND                    |
| Azuki bean | ND                    |
| Pea        | 2.4                   |

<sup>a</sup> Averages of duplicate determinations are recorded relative to SPI (Fujipro E = 100).

Raiden, Shiotsurunoko, York and Hill, with anti-glycinin antibodies. The reactivity varied with variety by less than 10%.

Table 3 shows the results of assays for several sources of food protein. In contrast to the high response against SPI, responses against lean pork and wheat gluten were negligible. Lean beef, hen's egg and milk casein gave no response. Only slight and negligible cross-reaction was detected with pea which bears, compared with other protein sources, a closer phylogenetic relationship with soya bean.

### Quantitation of soya protein in model sausage

Table 4 gives the results of an ELISA analysis of model sausages containing 0 to 20.8% SPI. Detected amounts were closely comparable to the actual amounts. The reference pork sausage, which contained no soya protein, showed no ELISA reading.

**TABLE 4**  
Determination by ELISA of soya protein  
content in pork sausages

| <i>SPI added<sup>a</sup></i> | <i>Soya protein detected<br/>by ELISA<sup>b</sup></i> |
|------------------------------|---|
| 0.0                          | ND  |
| 10.4                         | 10.7 ± 0.6  |
| 20.8                         | 20.9 ± 1.0  |

<sup>a</sup> Calculated levels from sausage recipe (% of total protein). Values represent SPI added.

<sup>b</sup> Observed levels in sausages (% of total protein). Each value represents mean ± SEM of triplicate samples.

## DISCUSSION

Studies on the immunochemical detection of soya protein have in the past been concerned primarily with structures expressed in intact proteins. Most determinants expressed in soya proteins appear to be of the conformational type, which are reported to be affected by their degree of denaturation. Previous observations showed that heat treatments are detrimental to the quantitative detection of proteins including soya protein. Of the major soya bean proteins glycinin exhibits the most heat-stable antigenicity (Koie and Djurtoft 1977), but even this is lost when heated to 120°C for 50 min (Günther *et al* 1977). Immunochemical methods are not considered to be ideal for the direct analysis of heated products, and their application is limited to qualitative screening of raw or mildly processed products (Hitchcock *et al* 1981; Griffiths *et al* 1984). Improved immunochemical methods utilise antibodies raised against soya proteins extracted under denaturing conditions and renatured by removing the denaturants. Such antibodies are then used to assay similarly renatured antigens (Koh 1978; Hitchcock *et al* 1981; Ravestein and Driedonks 1986). Even these and other advanced procedures give different responses according to the composition and the degree of protein denaturation of protein fractions.

The ELISA procedure established in this study is based on an antigenic structure which is a fragmental sequence of the soya protein, thereby offering highly specific detection and quantitation independently of its degree of denaturation. To the authors' knowledge this is the first use of an antigenic peptide fragment in quantitative immunoassay of soya proteins.

Results of the purification studies showed that autoclaving of glycinin for 3 h at 120°C followed by tryptic hydrolysis liberates several immunoreactive peptides. The antigenic determinants borne by these peptides should be of the sequential type in view of their resistance to these treatments (Maurer and Callahan 1980).

The lower limit of detection by the ELISA described in this report was about 4 g kg<sup>-1</sup> of total protein. By the previous methods (Parsons and Lawrie 1972; Bailey *et al* 1978; Poli *et al* 1979) it was difficult to detect the denatured soya

protein mixed at the level of lower than  $100 \text{ g kg}^{-1}$  of total protein. The ELISA signals obtained in this study could be regarded as showing the concentration of soya proteins or, to be more precise, as reflecting the level of antigenic determinant(s) on the fraction S-2-A-6 peptide whose amino acid sequence is part of the glycinin molecule and remains intact after autoclaving and tryptic digestion.

The ELISA response was not jeopardised by cross-reactivity of other non-soya protein food materials (Table 3). The seeds of many leguminous plants have been shown to contain proteins of similar subunit structure which show sequential homology to soya bean glycinin. Hence, cross-reactivity with azuki beans and pea might be expected, but the results presented show that these phylogenetically related legumins gave only negligible responses to soya glycinin antibody.

The ELISA response was independent of soya bean cultivar (Table 2). Recent knowledge acquired on the subunit composition (Mori *et al* 1981; Staswick *et al* 1981; Nielsen 1984) and the amino acid sequences of glycinin (Nielsen 1984; Staswick *et al* 1984; Momma *et al* 1985) help to identify sequence heterogeneity at several sites among these subunits and differences in their composition among the soya bean cultivars. The epitope of the S-2-A-6 peptide thus appears probably to locate in position(s) whose amino acid sequence is common among soya bean cultivars tested in this study. One may also consider that the differences in the immunoreactivity, if present, may arise from the difference in the content of glycinin in total soya proteins.

Quantitative analysis of soya proteins in pork sausage agreed well with the actual content (Table 4). Although analysis of soya proteins in the other models was not carried out in the present study, the procedure involved in the treatment of the sample does not appear to compromise the antigenicity of glycinin in different types of food product. Many processing steps that are liable to change the tertiary structure of proteins, eg sterilisation, cooking and extrusion, will not affect the immunoreactivity of the unique peptide. It thus appears that the ELISA procedure with the unique peptide as antigenic probe, as established here, is very reliable for the detection of soya proteins in mixed products, even if not acceptable for the general purposes of food product surveillance. The amino acid sequence of the epitope concerned in this study will be reported elsewhere.

## REFERENCES

- Agater I B, Briant K J, Llewellyn J W, Sawyer R, Bailey F J, Hitchcock C H S 1986 The determination of soya and meat protein in raw and processed meat products by specific peptide analysis. An evaluation. *J Sci Food Agric* **37** 317–331.
- Armstrong D J, Richert A H, Rieman S M 1982 The determination of isolated soya protein in raw and pasteurized meat products. *J Food Technol* **17** 327–337.
- Bailey F J 1976 A novel approach to the determination of soy protein in meat products using peptide analysis. *J Sci Food Agric* **27** 827–830.
- Bailey F J, Llewellyn J W, Hitchcock C H S, Dean A C 1978 The determination of soy protein in meat products using peptide analysis and the characterization of the specific soya peptide used in the calculations. *Chem & Ind* 477–478.
- Coomaraswamy M, Flint F O 1973 The histochemical detection of soya 'Novel Proteins' in comminuted meat products. *Analyst* **98** 542–545.

- Eldridge A C 1981 Determination of soya protein in processed foods. *J Amer Oil Chem Soc* **58** 483–485.
- Eldridge A C, Holmes L G 1979 Evaluation of a fluorometric technique for quantitative determination of soy flour in meat–soy blends. *J Food Sci* **44** 763–764.
- Engvall E 1980 Enzyme immunoassay ELISA and EMIT. *Methods Enzymol* **70** 419–439.
- Flint F O, Meech M U 1979 Quantitative determination of texturized soya protein by a sterological technique. *Analyst* **103** 252–258.
- Formo M W, Honold G R, MacLean D B 1974 Determination of soy products in meat–soy blends. *J Assoc Offic Anal Chem* **57** 841–846.
- Griffiths N M, Billington M J, Crimes A A, Hitchcock C H S 1984 An assessment of commercially available reagents for an enzyme-linked immunosorbent assay of soy protein in meat products. *J Sci Food Agric* **35** 1255–1260.
- Günther H O, Schweiger A, Baudner S 1977 Detection of plant proteins in meat products II. Recent developments. *Ann Nutr Alim* **31** 229–230.
- Guy R C E, Jayaram R, Wilcox C J K 1973 Analysis of commercial soya additive in meat products. *J Sci Food Agric* **24** 1551–1563.
- Hitchcock C H S, Bailey F J, Crimes A A, Dean B A G, Davis P J 1981 Determination of soya protein in food using an enzyme-linked immunosorbent assay procedure. *J Sci Food Agric* **32** 157–165.
- Kamm L 1970 Immunochemical quantitation of soybean protein in raw and cooked meat products. *J Assoc Offic Anal Chem* **53** 1248–1252.
- Koh T Y 1978 Immunochemical method for the identification and quantitation of cooked or uncooked beef and soya protein in mixture. *J Inst Can Sci Technol Aliment* **11** 124–128.
- Koie B, Djurtoft R 1977 Changes in the immunochemical response of soybean protein as a result of heat treatment. *Ann Nutr Alim* **31** 183–186.
- Koshiyama I 1972 Purification and physico-chemical properties of 11S globulin in soybean seeds. *Int J Peptide Protein Res* **4** 167–176.
- Lee Y B, Rickansrud D A, Hagberg E C, Briskey E J 1975 Quantitative determination of soya protein in fresh and cooked meat–soy blends. *J Food Sci* **40** 380–383.
- Lindqvist B, Ostgren J, Lindberg I 1975 A method for the identification and quantitative investigation of denatured protein in mixtures based on computer comparison of amino acid patterns. *Z Lebensm Unters-Forsch* **159** 5–22.
- Llewellyn J N, Deam A C, Sawyer R, Bailey F J, Hitchcock C H S 1978 The determination of meat and soya protein in meat products by peptide analysis. *J Food Technol* **13** 249–252.
- Maurer P H, Callahan H J 1980 Proteins and polypeptides as antigens. *Methods Enzymol* **70** 49–70.
- Medina M B 1988 Extraction and quantitation of soy protein in sausages by ELISA. *J Agric Food Chem* **36** 766–771.
- Medina M B, Phillips J G 1982 Investigations on trypsin-hydrolyzed peptides for protein identification. *J Agric Food Chem* **30** 1250–1253.
- Momma T, Negoro T, Hirano H, Matsumoto A, Uda K, Fukazawa C 1985 Glycinin A<sub>5</sub>A<sub>4</sub>B<sub>3</sub> mRNA:cDNA cloning and nucleotide sequencing of a splitting storage protein subunit of soybean. *Eur J Biochem* **149** 491–496.
- Mori T, Utsumi S, Inaba H, Kitamura K, Harada K 1981 Differences in subunit composition of glycinin among soybean cultivars. *J Agric Food Chem* **29** 20–23.
- Nielsen N C 1984 The chemistry of legume storage proteins. *Phil Trans Royal Soc Lond B* **304** 287–296.
- Olsman W J, Hitchcock C 1980 Detection and determination of vegetable proteins in meat products. In: *Developments in Food Analysis Techniques—2*, ed King R D. Applied Science, London, pp 255–260.
- Olsman W J, Dobbelaere S, Hitchcock C H S 1985 The performance of an SDS PAGE and an ELISA method for the quantitative analysis of soya protein in meat products: an international collaborative study. *J Sci Food Agric* **36** 499–507.
- Parsons A L, Lawrie R A 1972 Quantitative identification of soya protein in fresh and heated meat products. *J Food Technol* **7** 455–492.

- Poli G, Balsari A, Ponti W, Cantoni C, Massarv L 1979 Crossover electrophoresis with indirect immunofluorescence in the detection of soy protein in heated meat products. *J Food Technol* **14** 483–491.
- Rakosky J Jr 1975 Soy products for the meat industry. *J Agr Food Chem* **18** 1005–1009.
- Ravesteyn P, Driedonks R A 1986 Quantitative immunoassay for soya protein in raw and sterilized meat products. *J Food Technol* **21** 19–32.
- Rittenburg J H, Adams A, Palmer J, Allen J C 1987 Improved enzyme-linked immunosorbent assay for determination of soy protein in meat products. *J Assoc Offic Anal Chem* **70** 582–587.
- Staswick P E, Hermodson M A, Nielsen N C 1981 Identification of the acidic and basic subunit complexes of glycinin. *J Biol Chem* **256** 8752–8755.
- Staswick P E, Hermodson M A, Nielsen N C 1984 The amino acid sequence of the A<sub>2</sub>B<sub>1a</sub> subunit of glycinin. *J Biol Chem* **259** 13424–13430.
- Thanh V H, Shibasaki K 1976 Major proteins of soybean seeds. A straightforward fraction and their characterization. *J Agric Food Chem* **24** 1117–1121.
- Wilcke H L, Bodwell C E, Hopkins D T, Altschul A M 1986 New protein foods: a study of a treatise. *Adv Food Res* **30** 331–385.



## **Rates of Free Fatty Acid Formation from Phospholipids and Neutral Lipids in Frozen Cape Hake (*Merluccius* spp) Mince at Various Temperatures**

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### **ABSTRACT**

*Hake mince was stored in frozen condition at  $-5^{\circ}\text{C}$ ,  $-18^{\circ}\text{C}$  and  $-40^{\circ}\text{C}$ . Samples were withdrawn periodically, and the lipids were extracted and analysed for free fatty acid and phosphorus (P) content. The free fatty acid contents were corrected for the presence of acidic phospholipids and converted into genuine free fatty acid contents.*

*The results at  $-5^{\circ}\text{C}$  and  $-18^{\circ}\text{C}$  showed an initial rapid surge of free fatty acid formation with a concomitant decrease in P content, followed by a second phase of slower free fatty acid generation and loss of lipid-P. At  $-40^{\circ}\text{C}$  only one phase of free fatty acid generation and loss of lipid-P was observed.*

*The rates of the enzymic hydrolyses of the phospholipids and the neutral lipids were calculated at the three temperatures. An Arrhenius plot showed that the decrease in rate of free fatty acid formation with decreasing temperature was greater for the phospholipids than for the neutral lipids. In the rapid and more important phase, the two rates were identical at about  $-12^{\circ}\text{C}$  while above this temperature the phospholipids hydrolysed faster and below it the neutral lipids. Activation energies for the enzymic hydrolyses were calculated.*

**Key words:** Hake mince, phospholipids, neutral lipids, *Merluccius* spp, free fatty acids, enzymic hydrolysis, rate of free fatty acid formation.

## INTRODUCTION

In a previous publication it was shown that the free fatty acids (FFA) formed during storage at  $-18^{\circ}\text{C}$  of frozen Cape hake mince originated from both phospholipids and neutral lipids (de Koning *et al* 1987). From the data obtained it was possible to calculate the rates of enzymic hydrolysis at  $-18^{\circ}\text{C}$  of phospholipids and neutral lipids.

Recent work at this institute has included two more storage tests of hake mince at  $-18^{\circ}\text{C}$ , four tests at  $-5^{\circ}\text{C}$  and two at  $-40^{\circ}\text{C}$ . The results of the analyses of the extracted mince lipids at periodic intervals together with the calculated rates of FFA formation from the phospholipids and the neutral lipids at  $-5^{\circ}\text{C}$ ,  $-18^{\circ}\text{C}$  and  $-40^{\circ}\text{C}$  are the subject of the present report.

Work of a similar nature on phospholipid hydrolysis in cod fillets at 20, 0,  $-14$ ,  $-22$  and  $-29^{\circ}\text{C}$  was carried out by Olley and Lovern (1960). However, these authors did not attempt to separate the FFA formation into the two contributing components, and their results were of such accuracy that only rough comparison of total FFA formation at the different temperatures was possible.

## EXPERIMENTAL

### Origin and preparation of the mince

Headed and gutted hake caught within the last 24 h were received at the Institute packed in ice. They were skinned, deboned and minced through a 5 mm screen. The mince was then thoroughly mixed by hand and stored in  $\sim 300$  g quantities in polythene bags at  $-5^{\circ}\text{C}$ ,  $-18^{\circ}\text{C}$  and  $-40^{\circ}\text{C}$ .

Four separate tests on different hake were carried out at  $-5^{\circ}\text{C}$ , three at  $-18^{\circ}\text{C}$  and two at  $-40^{\circ}\text{C}$ . A total of five different hake batches caught at different times of the year and having lipid contents varying from 8.0 to 21.6 g kg $^{-1}$  were used. Two batches consisted of *Merluccius paradoxus* Franca and three of *Merluccius capensis* Castelnau. These data are summarised in Table 1.

Tests at  $-5^{\circ}\text{C}$  lasted about 70 days, tests at  $-18^{\circ}\text{C}$  about 300 days and tests at  $-40^{\circ}\text{C}$  for 711 and 245 days.

### Extraction and purification of the lipids

At specified intervals a weighed sample of frozen hake mince was extracted three times with excess chloroform/methanol (2:1 v) as described (de Koning 1966). The extracted lipids were purified according to the method of Folch *et al* (1957) and dried over anhydrous sodium sulphate, and the chloroform was removed *in vacuo* in a rotary evaporator. The lipids were taken up in a small amount of hexane, filtered again and dried under vacuum (4 Pa) on a freeze dryer, weighed and analysed for FFA and phosphorus (P) content. The phospholipid content of the lipids was calculated by multiplying the P content by 100/3.65 (de Koning *et al* 1987).

### Free fatty acid content

The FFA content of the extracted lipids was determined by titration with 0.02 M

TABLE 1  
Species of hake used, lipid content and lipid composition

| Temperature of storage | Test number | Hake species       | Lipid content (g kg <sup>-1</sup> wet mince) | Lipid composition |                    |
|------------------------|-------------|--------------------|--|-------------------|--------------------|
|                        |             |                    |  | Phospholipids (%) | Neutral lipids (%) |
| -5°C                   | 1           | <i>M paradoxus</i> | 8.0  | 67.1              | 32.9               |
|                        | 2           | <i>M capensis</i>  | 15.8   | 35.1              | 64.9               |
|                        | 3           | <i>M paradoxus</i> | 21.6   | 23.2              | 76.8               |
|                        | 4           | <i>M capensis</i>  | 12.3   | 41.5              | 58.5               |
| -18°C                  | 5           | <i>M capensis</i>  | 14.0   | 34.8              | 65.2               |
|                        | 6           | <i>M capensis</i>  | 15.8   | 35.1              | 64.9               |
|                        | 7           | <i>M capensis</i>  | 12.3   | 41.5              | 58.5               |
| -40°C                  | 8           | <i>M paradoxus</i> | 8.0  | 67.1              | 32.9               |
|                        | 9           | <i>M capensis</i>  | 15.8   | 35.1              | 64.9               |

sodium hydroxide against phenolphthalein in ethanol and expressed as oleic acid. The values were corrected for the presence of acidic phospholipids and converted into genuine FFA contents after P determination (de Koning *et al* 1987).

### Phosphorus content

The P content of the lipids was determined by a convenient method developed at this institute. A suitable amount of the lipid (about 100 mg) was hydrolysed in 50.0 ml 2 M hydrochloric acid or 1 M sulphuric acid at 120°C for 168 h, and after filtration the inorganic phosphate was determined in an aliquot by molybdenum blue development (de Koning and Mol 1989).

## RESULTS AND DISCUSSION

In Table 2 are presented the genuine FFA and P contents of the lipids extracted from frozen hake mince at progressive intervals of storage at various low temperatures.

By expressing the FFA and P contents in mmol kg<sup>-1</sup> lipid and using the fact (Olley and Lovern 1960) that, for each atom of lipid-P lost, two molecules of fatty acid are produced, it is possible to calculate the contribution from both the neutral lipids and the phospholipids to the total FFA produced. Inspection of the results shows that both lipids contributed to the FFA, but the relative contribution of each varied with the lipid composition, the storage temperature and the storage time. This is in contrast to the conclusion reached by Viswanathan Nair and Gopakumar (1985) who found that in frozen-stored milk fish the neutral lipid hydrolysis followed only after hydrolysis of the phospholipids. It is also in contrast to the results of Hardy *et al* (1979) who found that, in frozen cod fillets, FFA originated from the phospholipids only. When comparing lipids from the identical mince, as,

TABLE 2

Pattern of FFA formation from phospholipids and neutral lipids of frozen hake mince on storage at various low temperatures (data expressed in mmol kg<sup>-1</sup> total lipid)

| Test | Storage    | FFA <sup>a</sup> | $\Delta$ FFA | P     | $\Delta$ P | $\Delta$ FFA ex pl <sup>b</sup> | $\Delta$ FFA ex nl <sup>c</sup> |
|------|------------|------------------|--------------|-------|------------|---------------------------------|---------------------------------|
|      | Temp. Days |                  |              |       |            |                                 |                                 |
| 1    | 0          | 58.5             | —            | 790.8 | —          | —                               | —                               |
|      | 11         | 357.4            | 298.9        | 652.0 | 138.8      | 277.6                           | 21.3                            |
|      | 18         | 551.4            | 492.9        | 610.1 | 180.7      | 361.4                           | 131.5                           |
|      | 26         | 694.3            | 635.8        | 574.6 | 216.2      | 432.4                           | 203.4                           |
|      | 42         | 866.7            | 808.2        | 493.9 | 296.9      | 593.8                           | 214.4                           |
|      | 72         | 1003.6           | 945.1        | 477.7 | 313.1      | 626.2                           | 318.9                           |
| 2    | 0          | 38.7             | —            | 413.8 | —          | —                               | —                               |
|      | 11         | 352.1            | 313.4        | 358.9 | 54.9       | 109.8                           | 203.6                           |
|      | 23         | 429.1            | 390.4        | 353.5 | 60.3       | 120.6                           | 269.8                           |
|      | 37         | 650.0            | 611.3        | 334.4 | 79.4       | 158.8                           | 452.5                           |
|      | 51         | 746.1            | 707.4        | 317.6 | 96.2       | 192.4                           | 515.0                           |
|      | 72         | 812.8            | 774.1        | 307.9 | 105.9      | 211.8                           | 562.3                           |
| 3    | 0          | 35.1             | —            | 275.8 | —          | —                               | —                               |
|      | 8          | 169.9            | 134.8        | 262.2 | 10.6       | 21.2                            | 113.6                           |
|      | 34         | 475.5            | 440.4        | 196.0 | 76.8       | 153.6                           | 286.8                           |
|      | 50         | 520.2            | 485.1        | 186.0 | 86.8       | 173.6                           | 311.5                           |
|      | 74         | 756.4            | 721.3        | 173.0 | 99.8       | 199.6                           | 521.7                           |
| 4    | 0          | 81.9             | —            | 488.7 | —          | —                               | —                               |
|      | 8          | 329.4            | 247.5        | 456.1 | 32.6       | 65.2                            | 182.3                           |
|      | 21         | 518.8            | 436.9        | 380.6 | 108.1      | 216.2                           | 220.7                           |
|      | 36         | 744.7            | 662.8        | 351.8 | 136.9      | 273.8                           | 389.0                           |
|      | 50         | 952.8            | 870.9        | 341.2 | 147.5      | 295.0                           | 575.9                           |
|      | 75         | 1151.8           | 1069.9       | 302.5 | 186.2      | 372.4                           | 697.5                           |
| 5    | 0          | 82.3             | —            | 409.9 | —          | —                               | —                               |
|      | 55         | 390.7            | 308.4        | 335.7 | 74.2       | 148.4                           | 160.0                           |
|      | 118        | 512.1            | 429.8        | 322.8 | 87.1       | 174.2                           | 255.6                           |
|      | 210        | 750.0            | 667.7        | 287.3 | 122.6      | 245.2                           | 422.5                           |
|      | 307        | 866.7            | 784.4        | 284.1 | 125.8      | 251.6                           | 532.8                           |
| 6    | 0          | 38.6             | —            | 413.8 | —          | —                               | —                               |
|      | 59         | 434.8            | 396.1        | 360.9 | 52.9       | 105.9                           | 290.2                           |
|      | 102        | 538.7            | 500.0        | 328.3 | 85.5       | 171.1                           | 328.9                           |
|      | 165        | 682.6            | 644.0        | 324.7 | 89.1       | 178.2                           | 465.8                           |
|      | 221        | 758.2            | 719.5        | 318.0 | 95.9       | 191.7                           | 527.8                           |
|      | 305        | 1147.5           | 1108.9       | 309.9 | 103.9      | 207.9                           | 901.0                           |
| 7    | 0          | 84.4             | —            | 479.7 | —          | —                               | —                               |
|      | 34         | 407.8            | 323.4        | 414.8 | 64.9       | 129.8                           | 193.6                           |
|      | 69         | 636.9            | 552.5        | 403.5 | 76.2       | 152.3                           | 400.1                           |
|      | 109        | 717.7            | 633.3        | 390.6 | 89.1       | 178.2                           | 455.2                           |
|      | 244        | 796.1            | 711.7        | 358.0 | 121.7      | 243.4                           | 468.3                           |
|      | 338        | 1006.4           | 922.0        | 349.9 | 129.8      | 259.5                           | 662.5                           |
| 8    | 0          | 56.7             | —            | 790.8 | —          | —                               | —                               |
|      | 199        | 151.4            | 94.7         | 761.8 | 29.0       | 58.0                            | 36.7                            |
|      | 317        | 199.3            | 142.6        | 746.0 | 44.8       | 89.6                            | 53.0                            |
|      | 711        | 419.5            | 362.8        | 712.4 | 78.4       | 156.8                           | 206.0                           |
| 9    | 0          | 38.6             | —            | 413.8 | —          | —                               | —                               |
|      | 117        | 93.6             | 55.0         | 409.9 | 3.9        | 7.8                             | 47.2                            |
|      | 245        | 176.6            | 138.0        | 406.1 | 7.8        | 15.5                            | 122.5                           |

<sup>a</sup> Values are corrected for the presence of acidic phospholipids.

<sup>b</sup> Generated from phospholipids =  $2 \times \Delta P$ .

<sup>c</sup> Generated from neutral lipids =  $\Delta FFA - 2\Delta P$ .

for example, in tests 1 and 8, it may be seen that at  $-5^{\circ}\text{C}$  the contribution of the phospholipids to the total FFA was roughly twice that of the neutral lipids whereas at  $-40^{\circ}\text{C}$  they were similar. In other words the rate of hydrolysis of the phospholipids declined more sharply with decreasing temperature than that of the neutral lipids. This may also be deduced from evaluation of the results in tests 2 and 6, 4 and 7 or 6 and 9.

By calculating and plotting regression lines as described previously (de Koning *et al* 1987) for the FFA formation from both lipids as a function of the storage time, it becomes clear that at  $-5^{\circ}\text{C}$  and at  $-18^{\circ}\text{C}$  there is an initial phase of rapid FFA formation from both lipids followed by a second phase of slower FFA formation. The rapid phase lasted about 25 days at  $-5^{\circ}\text{C}$  and about 60 days at  $-18^{\circ}\text{C}$ . In contrast, at  $-40^{\circ}\text{C}$  no such two separate phases were observed. The rapid phase of FFA production at  $-5$  and  $-18^{\circ}\text{C}$  is, from a commercial point of view, more important than the slow phase since most of the texture deterioration of the mince takes place during that period (de Koning *et al* 1986). By using the slopes of the regression lines and the lipid compositions (see Table 1) the rates of hydrolysis of the phospholipids and neutral lipids at the different temperatures may now be calculated. The results of the calculations are recorded in Table 3. Inspection of the results at  $-5^{\circ}\text{C}$  shows that no appreciable differences are apparent in the respective rates for the *M paradoxus* and *M capensis* species.

A plot of the logarithm of the two reaction rates ( $\log k$ ) in the initial rapid phase of the reaction versus the reciprocal of the absolute temperature ( $1/T$ ) is shown in Fig 1. Mathematical expressions for the two regression lines in the rapid phase are:

$$\log k_{\text{phospholipids}} = 13.21 - \frac{3346}{T} \quad r = -0.9886 \quad n = 9$$

$$\log k_{\text{neutral lipids}} = 8.95 - \frac{2334}{T} \quad r = -0.9702 \quad n = 9$$

where  $r$  is the correlation coefficient and  $n$  is the number of tests.

From this plot it may be readily deduced that at a temperature of 261.0 K ( $-12^{\circ}\text{C}$ ) the rates of hydrolysis for the phospholipids and neutral lipids in the rapid phase are identical, while above this temperature the phospholipids hydrolyse faster than the neutral lipids and slower below this temperature. This leads to the general conclusion, other variables being equal, that above  $-12^{\circ}\text{C}$  lean fish should develop a high FFA level in their total body lipids more rapidly than fatty fish while the reverse should apply below  $-12^{\circ}\text{C}$ .

The slopes of the regression lines allow calculation of the activation energies for the two enzymic hydrolyses (Fruton and Simmonds 1953). These activation energies are  $66.03 \text{ kJ mol}^{-1}$  and  $44.73 \text{ kJ mol}^{-1}$  for the phospholipids and neutral lipids respectively.

Similar calculations for the FFA formation in the slow phase yield the following mathematical expressions for the reaction rates:

$$\log k_{\text{phospholipids}} = 8.75 - \frac{2435}{T} \quad r = -0.9656 \quad n = 9$$

TABLE 3  
Rates of FFA formation from hake mince phospholipids and neutral lipids at various temperatures (values expressed as mmol FFA day<sup>-1</sup> kg<sup>-1</sup> respective lipid)

| Temperature | Test | Hake species       | Rapid phase <sup>a</sup> |                | Slow phase <sup>a</sup> |                |
|-------------|------|--------------------|--------------------------|----------------|-------------------------|----------------|
|             |      |                    | Phospholipids            | Neutral lipids | Phospholipids           | Neutral lipids |
| -5°C        | 1    | <i>M paradoxus</i> | 24.7                     | 24.9           | 5.7                     | 7.9            |
|             | 2    | <i>M capensis</i>  | 14.8                     | 17.9           | 5.4                     | 8.6            |
|             | 3    | <i>M paradoxus</i> | 20.3                     | 10.4           | 4.7                     | 8.1            |
|             | 4    | <i>M capensis</i>  | 25.1                     | 16.8           | 6.8                     | 15.2           |
| -18°C       | 5    | <i>M capensis</i>  | 7.7                      | 4.5            | 1.3                     | 2.3            |
|             | 6    | <i>M capensis</i>  | 5.1                      | 7.6            | 1.0                     | 3.7            |
|             | 7    | <i>M capensis</i>  | 5.4                      | 9.8            | 1.0                     | 1.4            |
| -40°C       | 8    | <i>M paradoxus</i> | 0.33                     | 0.88           | —                       | —              |
|             | 9    | <i>M capensis</i>  | 0.18                     | 0.77           | —                       | —              |

<sup>a</sup> See text.

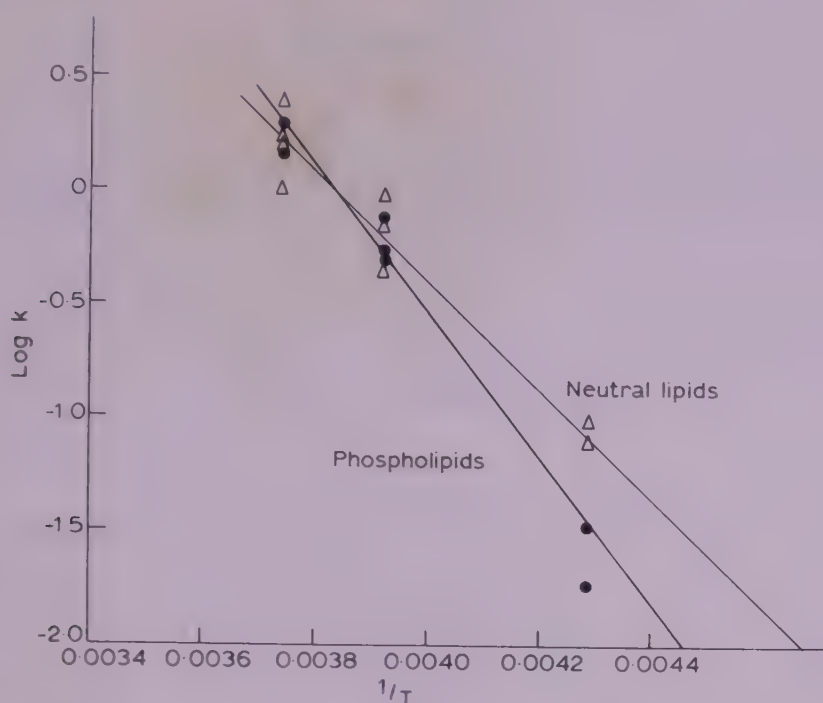


Fig 1. Arrhenius plot of the enzymic hydrolysis of phospholipids and neutral lipids in hake mince during the rapid phase of the reaction.

and

$$\log k_{\text{neutral lipids}} = 7.00 - \frac{1906}{T} \quad r = -0.9186 \quad n = 9$$

From these regression lines it can now be readily concluded that, when the mince has reached the slow reaction phase, the neutral lipids hydrolyse faster than the phospholipids over the whole of the frozen temperature range.

The slopes of the regression lines, and therefore the two activation energies in the slow phase, are similar and are calculated to be 46.66 and 36.52 kJ mol<sup>-1</sup> for the phospholipids and neutral lipids, respectively. Using the data presented in this report it becomes possible to calculate roughly the FFA content attained by hake mince during a known period of frozen storage at a known temperature by determining its lipid content and composition at the outset of the storage. Similarly, it is possible to calculate roughly the time spent by hake mince in frozen storage at a specific temperature by determining the FFA content and composition of its lipids.

Finally, it should be mentioned that no essential difference has been observed at this institute in the rate of FFA formation of hake mince and hake fillets. However, the difficulty with the analytical data for hake fillets is that they do not lend themselves to the rigorous type of calculation applicable to mince, since the material analysed at the different storage periods is not uniform (de Koning *et al* 1986).

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## REFERENCES

- De Koning A J 1966 Phospholipids of marine origin. I. The hake (*Merluccius capensis*, Castelnau). *J Sci Food Agric* **17** 112–117.
- De Koning A J, Mol T. 1989 A convenient method for the accurate determination of phosphorus in fish oils. *Fat Sci Technol* **91** 36–38.
- De Koning A J, Milkovitch S., Mol T. 1986 Quantitative quality tests for frozen fish. Part IIA. Changes in the proteins and lipids of frozen hake fillets and mince stored at  $-18^{\circ}\text{C}$ . *Fishing Ind Res Inst Tech Rep* **1** 89–96.
- De Koning A J, Milkovitch S, Mol T 1987 The origin of free fatty acids formed in frozen Cape hake mince (*Merluccius capensis*, Castelnau) during cold storage at  $-18^{\circ}\text{C}$ . *J Sci Food Agric* **39** 79–84.
- Folch J, Lees M, Sloane S G H 1957 A simple method for the isolation and purification of the total lipides from animal tissue. *J Biol Chem* **226** 497–509.
- Fruton J S, Simmonds S 1953 *General Biochemistry* (1st edn). John Wiley and Sons, New York, p 254.
- Hardy R, McGill A S, Gunstone F D 1979 Lipid and autoxidative changes in cold stored cod (*Gadus morhua*). *J Sci Food Agric* **30** 999–1006.
- Olley J, Lovern J A 1960 Phospholipid hydrolysis in cod flesh stored at various temperatures. *J Sci Food Agric* **11** 644–652.
- Viswanathan Nair P G, Gopakumar K 1985 Selective release of fatty acids during lipid hydrolysis in frozen-stored milk fish (*Chanos chanos*). *Fish Technol Ind* **22** 1–4.

## Behaviour of Aroma Volatiles during the Evaporative Concentration of Some Tropical Fruit Juices and Pulps

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### ABSTRACT

*The dynamics of aroma separation from some tropical fruit juices and pulps was studied using a thin film evaporator. In the case of mango (Mangifera indica Linn, Alphonso and Totapuri varieties) and guava (Psidium guajava Linn) pulps, 90 % of each of the volatile fraction, esters, carbonyls and alcohols, was removed at 30–35 % evaporation of the water in a single pass. In the case of pineapple (Ananas sativa Schult F) juice, 90 % separation of esters and carbonyls required about 80 % and 47 % evaporation, respectively. However, the behaviour of alcohols in pineapple juice was found to be similar to that of mango and guava. Mango pulp from Alphonso and Totapuri varieties on 60 % evaporation showed a loss in oxygenated terpenes of 80 % and 63 %, respectively, while guava pulp lost about 58 % oxygenated terpenes on 60 % concentration.*

*The dynamics of the total aroma volatiles separation from the fruit juices and pulps showed that, in a single pass evaporation, 85–90 % volatiles were removed at a juice evaporation degree of 60 % water, while the same extent of aroma removal occurred at 32–35 % total evaporation by multiple pass.*

**Key words:** Fruit aroma separation, fruit juice concentration, tropical fruits.

### INTRODUCTION

The aroma complex of a fruit is usually a mixture of a large number of volatile organic compounds, the individual aroma components differing according to their

boiling points, solubility and molecular structure and above all according to their relative volatility. Because of the complexity of fruit aroma volatiles, it is rather difficult to separate aroma volatiles from different fruit juices/pulps using one and the same aroma recovery plant with the same effect, ie to separate, rectify and concentrate the aroma. For each type of fruit juice a specific degree of juice evaporation is required in order to recover almost all the aroma. The amount of water that must be evaporated in order to achieve the desired degree of aroma removal has been reported for several temperate fruits (Eisenhardt *et al* 1958; Pilnik and Zwikker 1962; Bomben *et al* 1967; Sulc *et al* 1968; Sulc 1970, 1978, 1980, 1984; Koch 1972).

Tropical fruit juice and pulp concentrates constitute major items of export from India. However, no published information is available in the case of tropical fruits on the dynamics of aroma separation in relation to the degree of juice evaporation. In this communication the results of a study on the dynamics of aroma separation from some commercially important juices and pulps are reported.

## EXPERIMENTAL

### Raw material

#### *Mango (Mangifera indica Linn)*

Mangoes of the Alphonso and Totapuri varieties purchased in the local market were ripened at room temperature ( $25 \pm 5^\circ\text{C}$ ) under natural ripening conditions.

#### *Guava (Psidium guajava Linn)*

Fully ripe guava (Allahabad variety) were purchased from the local market.

#### *Pineapple (Ananas sativa Schult F)*

Pineapples purchased from the local market were ripened at room temperature under natural ripening conditions.

### Pulp/juice extraction

#### *Mango*

Fully ripe mangoes (100 kg) of each variety were washed in tap water, the stem portion and any black specks on the surface were removed by a stainless steel knife, the fruits were cut into slices, and the pulp was extracted by passing the cut slices through an APV pulper, Senior model (Aluminium Plant and Vessels Co Ltd, London, UK), fitted with a 0.6 mm dia sieve.

#### *Guava*

Firm ripe fruits (100 kg) were washed in tap water, peeled by dipping in boiling NaOH solution ( $25 \text{ g litre}^{-1}$ ) for 2 min and washed in water. Any adhering peel was removed by scrubbing, and the fruits were dipped in citric acid solution ( $5 \text{ g kg}^{-1}$ ) for 1 min to neutralise any residual alkali on the surface and washed in water to remove excess acid. Specks were removed with a stainless steel knife and the fruits were crushed in a fruit mill. The pulp was extracted by passing the crushed mass through the APV pulper as above.

### Pineapple

Ripe pineapple fruits (100 kg) were selected and the crown and stem portions were removed and washed in tap water. The fruits were sliced using a mechanical slicer and the rind was removed either with a knife or with punches. The prepared slices were crushed in a fruit mill. The crushed mass was pasteurised at 85°C and spun in a basket centrifuge, and the clear pineapple juice was collected.

### Determination of dynamics of aroma separation

A thin film evaporator (Turba film, Votator Division, Chemetron Corp, Louisville, KY, USA) was used to study the relationship between extent of evaporation and degree of aroma removal. To study the aroma separation dynamics in a single pass evaporation, 5-kg batches of juice/pulp were passed through the evaporator and the aroma-containing vapours were condensed with chilled water at 2°C and collected. With adjustments in feed rates it was possible to evaporate the juice/pulps to the desired degree of evaporation. For studying aroma separation dynamics in multipass evaporation, 10 kg of juice or pulp was passed through the evaporator to evaporate about 10% water in each pass. The same juice or pulp was repeatedly passed through the evaporator till the desired final degree of evaporation was achieved. The condensate obtained in each pass was collected. The combined aroma condensates were analysed for their volatile components by chemical methods. Total volatiles and groups of aroma components in the fruit juice and pulps were determined by analysing the distillates obtained by steam distilling the samples to remove all the volatiles.

The operating conditions of the thin film evaporator used in this study are given in Table 1.

In order to achieve the desired extent of water evaporation the feed rates of the pulps and juice were varied, keeping the steam pressure constant.

### Analysis

The aroma distillates were analysed for esters (Leonard and Willard 1960),

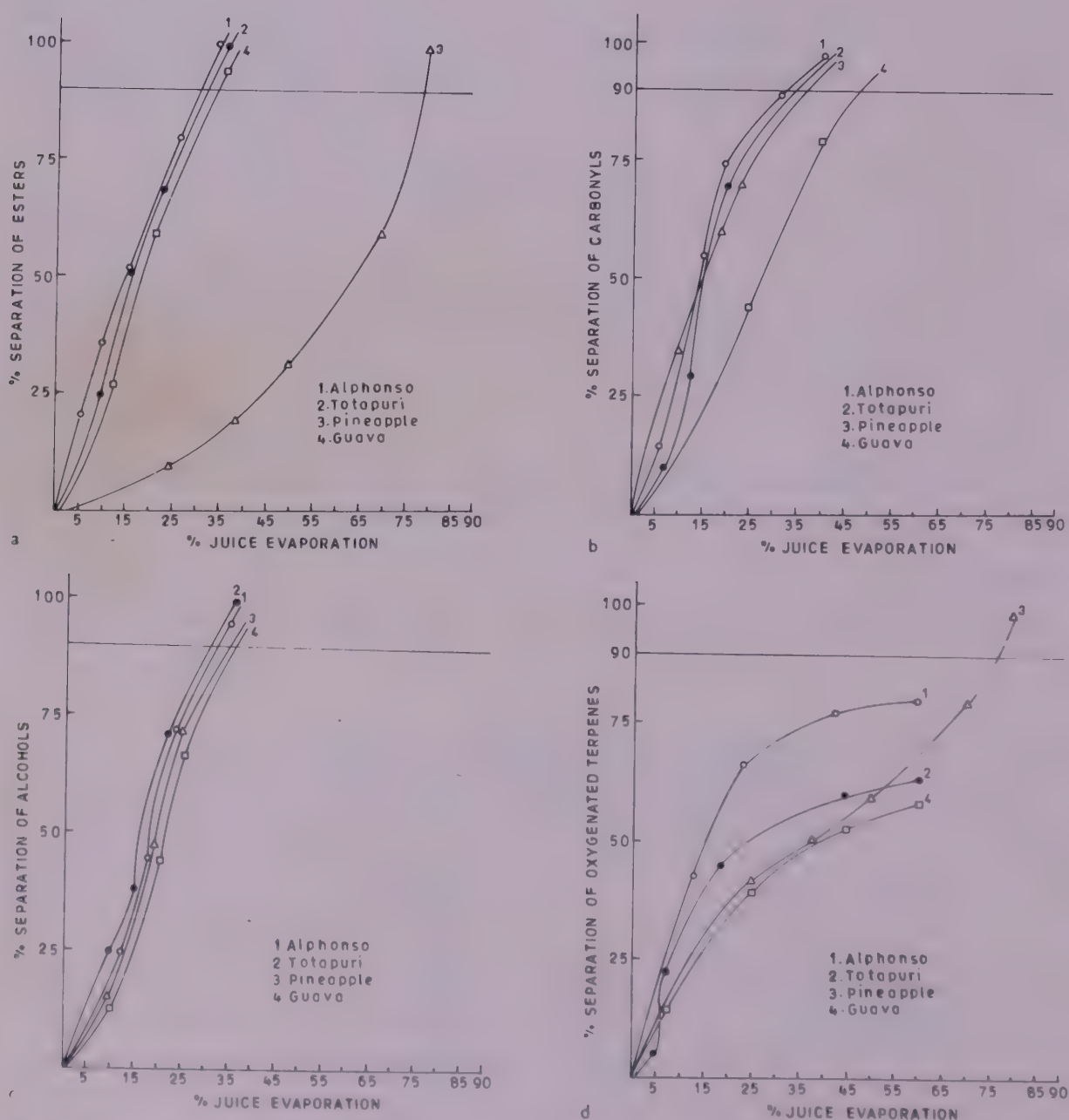
**TABLE 1**  
Operating conditions of the thin film evaporator

| <i>Parameters</i>                 | <i>Values/details</i>                   |
|-----------------------------------|---|
| Operating pressure                | 9599 Pa                                 |
| Type of thin film evaporator      | Turba film, gravity flow                |
| Capacity of the evaporator        | 0.083 m <sup>2</sup> heat transfer area |
| Heating medium for the evaporator | Steam                                   |
| Steam pressure                    | 1 kg cm <sup>-2</sup>                   |
| Steam temperature                 | 120°C                                   |
| Flow rate                         | 15–18 kg h <sup>-1</sup>                |
| Feed rates                        |   |
| Mango pulp                        | 24–143 kg h <sup>-1</sup>               |
| Guava pulp                        | 23–135 kg h <sup>-1</sup>               |
| Pineapple juice                   | 20–170 kg h <sup>-1</sup>               |

carbonyls (Lappan and Clark 1951), alcohols (Snell *et al* 1953), total oxygenated terpenes (Latrasse *et al* 1982) and chemical oxygen demand (COD, Dougherty 1968).

## RESULTS AND DISCUSSION

Figures 1 and 2 show the dynamics of aroma separation from the different fruit juices and pulps studied. In the case of mango (Alphonso and Totapuri) and guava pulps, 90% of each of the volatile fraction, esters (Fig 1a), carbonyls (Fig 1b) and alcohols (Fig 1c), can be separated at a juice evaporation degree of 30–35%. In the



**Fig 1.** Effect of degree of evaporation on the separation of total volatiles and groups thereof from mango (Alphonso and Totapuri) and guava pulp and pineapple juice. (a) Volatile esters; (b) carbonyls; (c) alcohols; (d) oxygenated terpenes.

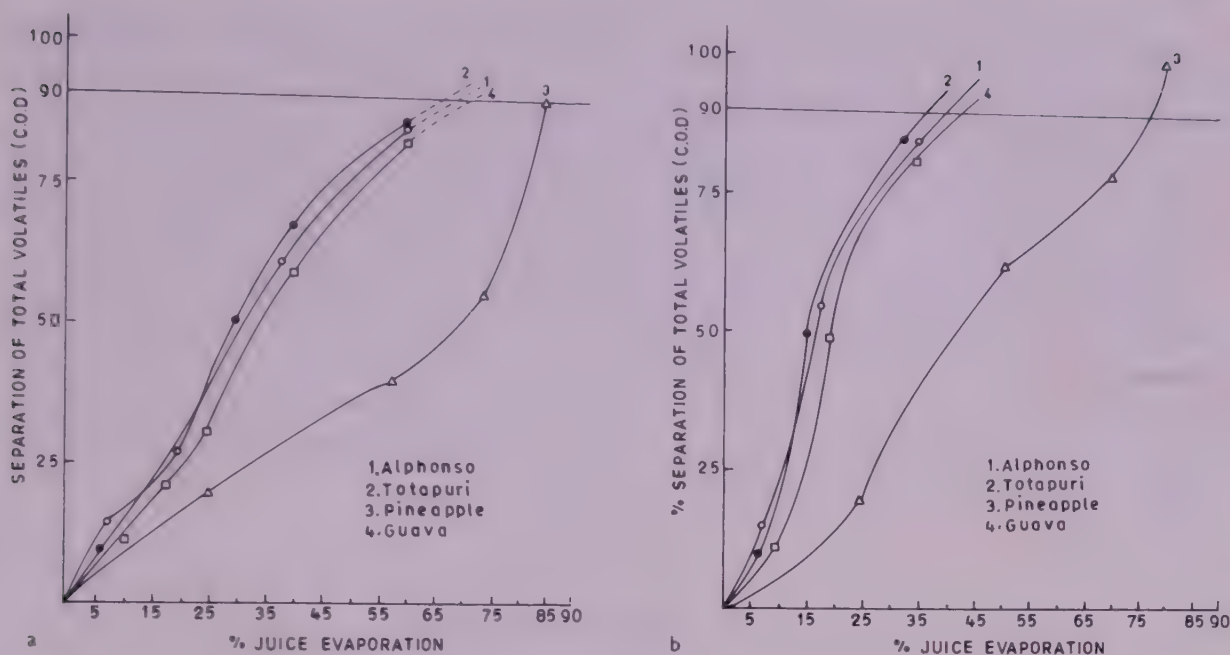


Fig 2. Relationship between percentages of water evaporated and aroma separated during (a) single-pass, and (b) multipass evaporation of mango and guava pulps and pineapple juice.

case of pineapple juice the separation of esters requires nearly 80% evaporation, whereas carbonyls are separated at 47% evaporation. However, the separation of alcohols follow a trend similar to that for mango and guava.

Figure 1d shows the separation dynamics of oxygenated terpene components from fruit pulps of mango (Alphonso and Totapuri), guava and pineapple juice. In commercial practice it is rather difficult to concentrate fruit pulps to more than about  $300 \text{ g kg}^{-1}$  total soluble solids because of the steep rise in viscosity during concentration. Therefore, in these studies no attempt was made to find out the dynamics of aroma separation from these pulps beyond 60% water evaporation.

Alphonso and Totapuri mango pulp on evaporation to remove 60% water showed a loss in oxygenated terpenes of 80% and 63%, respectively. Guava pulp lost about 58% oxygenated terpenes on concentration to a similar level. Based on these results it can be seen that in mango pulp substantial amounts of oxygenated terpene compounds are retained on twofold concentration. Oxygenated terpenes have been shown to be important aroma compounds in some mango varieties (Bandyopadhyay and Golap 1979; Abd-El-Baki *et al* 1981; Macleod and Pieris 1984). This finding substantiates the experience that the concentrate has sufficiently strong aroma and other organoleptic qualities on conversion to beverages. The dynamics of total aroma separation for mango (Alphonso and Totapuri), guava and pineapple are represented in Figs 2a and b. The curves in Fig 2a for mango and guava pulps are extrapolated to 90% aroma separation.

Figure 2a shows the aroma separation dynamics when the juice/pulps were concentrated to different levels in a single pass, and Fig 2b shows the dynamics of aroma separation when the juice/pulps were concentrated by multipass evaporation. It can be seen that aroma removal from the fruit pulps and juice is much more efficient when the juice/pulps were multipassed as compared with a

single pass. For example, in a single-pass evaporation of mango and guava pulps at a juice evaporation of 60% water, about 85 to 90% volatiles were lost whereas, when the fruit juice/pulps were passed repeatedly, the same extent of aroma loss occurred at a juice evaporation level of 32 to 35% water. The lower loss of aroma in single-pass evaporation could be due to non-equilibrium evaporation (Thijassen and Van Oyen 1977). This shows that, by employing juice evaporated in a single pass, it is possible to retain more aroma in the product than when juice or pulps are concentrated by multipass. Pineapple juice shows a similar trend in aroma separation by single and multipass. However, since thin juices such as that of pineapple are usually concentrated to about  $700 \text{ g kg}^{-1}$  of total soluble solids (more than 80% water evaporation), losses in aroma volatiles in the concentrate prepared either by single or multipass would be similar.

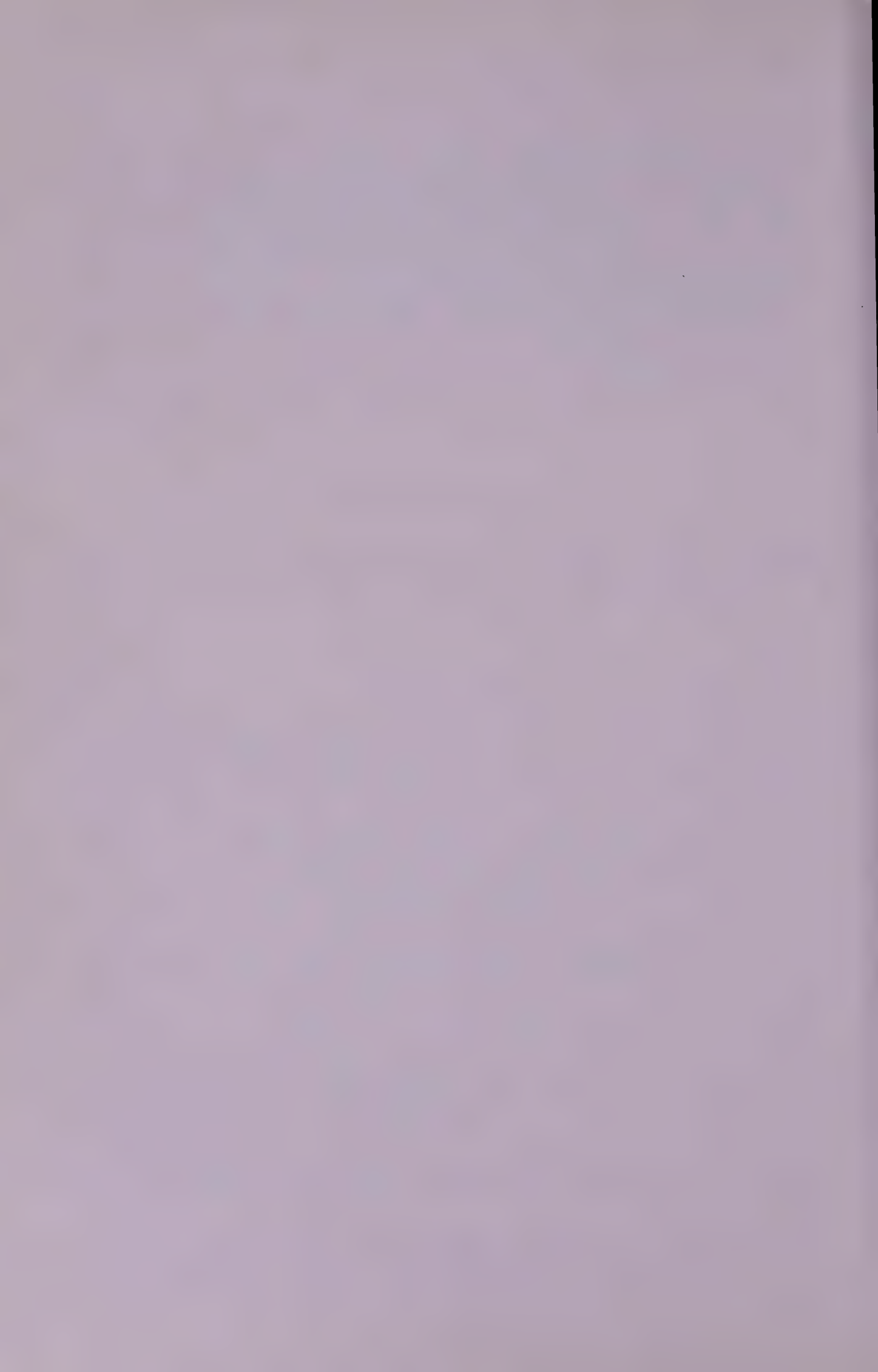
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### REFERENCES

- Abd-El-Baki M M, Askar A, El-Samahy S K, Abd-El Fadeel M G 1981 Studies on mango flavour. *Dtsch Lebensm Rdsch* **77** 139.
- Bandyopadhyay C, Golap A S 1979 Quantitation of flavour notes in mango varieties. In: *Proc First Indian Convention of Food Scientists and Technologists*, ed Achaya K T. AFST, Mysore, p 4.
- Bomben J L, Mannheim H G, Morgan A I 1967 Operating conditions for aroma recovery by new vacuum stripping method and evaluation of aroma solutions. *Fruchtsaft Ind* **12** 44-53.
- Dougherty M M 1968 A method for measuring the water soluble volatile constituents of citrus juices and products. *Food Technol* **22** 1455-1456.
- Elsenhardt N H, Eskew R K, Claffey H B, Aceto N C 1958 The preparation of full flavour berry juice concentrate. *US Dept Agric ARS-73-20*.
- Koch J 1972 Zur Technologie der Fruchtsaftherstellung. *Flussiges Obst* **39** 52-66.
- Lappan G R, Clark L C 1951 Colorimetric method for determination of traces of carbonyl compounds. *Anal Chem* **23** 541-542.
- Latrasse A, Lantin B, Mussilon P, Sarris J 1982 Aroma quality of raspberry. I. Rapid colorimetric determination of an aroma index using vanillin in concentrated sulphuric acid. *Lebensm Wiss Technol* **15** 19-21.
- Leonard R M, Willard B R 1960 Changes in volatile constituents during baking of sherry wine by Tressler process. *Food Technol* **14** 30-33.
- Macleod A J, Pieris N M 1984 Comparison of volatile components of some mango cultivars. *Phytochem* **23** 361-366.
- Pilnik W, Zwicker P 1962 Volatile fruit flavours. *Report of the Scientific and Technical Commission of the International Federation of Fruit Juice Producers Union*, Juris, Zurich, pp 405-408.
- Snell F D, Snell C T, Snell C A (eds) 1953 Primary and secondary alcohols. *Colorimetric Methods of Analysis*. Van Nostrand, Princeton, NJ, p 39.
- Sulc D 1970 Dynamik der Fruchtaromaseparierung. *Flussiges Obst* **37** 135-144.

- Sulc D 1978 Dynamics of separation of aromatic substances from fruit juices. *Masinstov u procesnoj tehnici Beograd* 289–309.
- Sulc D 1980 Verfahrenstechnische Aspekte bei der Konzentrierung und Aromaseperierung von Fruchtsäften. *Int Fruchtsaft Union, Ber Wiss Techn Komm* 16 1–21.
- Sulc D 1984 Fruit juice concentration and aroma separation. *Confructa* 28 258–318.
- Sulc D, Curakovic M, Nikolajevic R 1968 Evaporation dynamics of flavour components during blackberry juice concentration. *Hemijska Industrija, Beograd* 5 866–871.
- Thijassen H A C, Van Oyen V S M 1977 Analysis and economic evaluations of concentration alternatives for liquid foods—quality aspects and cost of concentrations. *J Food Engng* 1 215–240.



## Composition and Odour of Volatiles from Autoxidised Methyl Arachidonate

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### ABSTRACT

*Methyl arachidonate was subjected to autoxidation at room temperature either alone or in a water emulsion. Volatiles in the headspace were collected on Tenax and analysed by GC–MS, and the amounts and identities of the major and minor volatiles were compared. Generally, the same compounds were formed in both systems, but whereas the amounts varied the changes were consistent with the role of water in lipid oxidation. Twelve volatiles accounted for 90% of the total, with oct-1-en-3-ol (30–40%), hept-2-enal (10–17%) and hexanal (6–17%) most prominent. Over 40 other volatiles were detected. The odour of individual components in both systems was assessed using a GC equipped with a sniffing port.*

**Key words:** Autoxidation, methyl arachidonate, odour, volatiles, emulsion.

### INTRODUCTION

The autoxidation of unsaturated fatty acids proceeds by a multistep reaction with hydroperoxides as the primary products. In the case of arachidonic acid (eicosa-5,8,11,14-tetraenoic acid) or its methyl ester, six hydroperoxides have been found (Terao and Matsushita 1981; Yamagata *et al* 1983) although it can be predicted that up to 13 different hydroperoxides are possible (Badings 1970). Further breakdown of these hydroperoxides can give rise to at least 50 different compounds but many of these are capable of further oxidation and/or reaction (Grosch 1982). The actual

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amounts and types of compound formed depend on the relative amounts of each hydroperoxide and the conditions under which autoxidation takes place. It is known that the rate of autoxidation is affected by a number of parameters such as surface area, oxygen pressure, water activity and temperature (Labuza 1971). It is likely that the same parameters also influence the nature and relative amounts of the autoxidation products although there are few definitive data.

The literature contains information on the volatiles formed during the autoxidation of methyl arachidonate (Ellis *et al* 1966) and arachidonic acid (Badings 1970), but only the carbonyl compounds were isolated and identified. Ellis *et al* (1966) examined aldehydes from the autoxidation of methyl arachidonate and found hexanal as the major aldehyde (62%) with significant amounts of heptanal (16%). Alkenals ( $C_7$ – $C_{11}$ ) and dienals ( $C_9$ – $C_{12}$ ) were also found. Badings (1970) extracted a mixture of aldehydes and ketones from autoxidised arachidonic acid and identified them by mass spectrometry of the dinitrophenyl derivatives. Hexanal, heptanal, nonan-2-one and undecan-2-one were the major compounds with significant amounts of other saturated and unsaturated carbonyl compounds.

The aim of the present study was to identify carbonyl and non-carbonyl volatiles from autoxidised methyl arachidonate by headspace sampling and GC-MS. In addition, the variation in oxidation products caused by the presence of water was studied, and the odour of individual components was assessed by sniffing the eluate from a modified gas chromatograph.

## EXPERIMENTAL

### Materials

Methyl arachidonate (methyl eicosa-5,8,11,14-tetraenoate, 99% pure) was purchased from Sigma Chemical Co, stored at  $-20^{\circ}\text{C}$  and used without further purification. Reagents and solvents were analytical or HPLC grade. Glassware was heated in an oven at  $150^{\circ}\text{C}$  for several hours prior to use to remove any trace volatiles.

### Autoxidation of methyl arachidonate

Extraction thimbles (Whatman;  $10 \times 50$  mm) were oven dried at  $80^{\circ}\text{C}$  for 30 min. After cooling, the closed end was cut off so that the resulting cylinder was a push fit over the central tube of a Dreschel head. To obtain methyl arachidonate in a finely divided form on the thimble, 150 mg was dissolved in diethyl ether (3 ml) and 1 ml of solution was applied to each of three thimbles. Ether was removed under a stream of nitrogen and the Dreschel heads with the thimbles were then mounted in screw-top 250-ml conical flasks using screw caps fitted with PTFE-lined seals. A Tenax trap made from glass-lined stainless-steel tubing (1/16 in od, 0.7 mm id; SGE Ltd, Milton Keynes) was fixed to the outlet of the Dreschel head but the inlet was left open to the atmosphere. The three flasks were set up and stored at room temperature ( $20$ – $22^{\circ}\text{C}$ ) in the dark for 48 h. To study the effect of water on autoxidation, an emulsion of methyl arachidonate (150 mg in 1.5 ml water) was formed by placing the mixture in an ultrasonic bath for 5 min. Portions equivalent

to 50 mg were then applied to each of three thimbles on Dreschel heads. These were mounted in flasks fitted with Tenax traps and treated as above.

### Headspace collection

After autoxidation, each flask was connected to a dry nitrogen supply and the headspace was flushed through the Tenax traps at 50–60 ml min<sup>-1</sup> for 30 min at room temperature (20–22°C). Headspace was collected simultaneously from the replicates. The traps were removed from the flasks and flushed for a further 2 min with dry nitrogen to remove any water. Prior to analysis, traps were stored in stoppered glass tubes at 4°C to minimise any loss of volatiles.

### Gas chromatography–mass spectrometry

Chromatography was performed on a Carlo Erba 4200 gas chromatograph equipped with a 'Unijector' (SGE Ltd) set in the concentrator-headspace mode and fitted with a CP52 wax column (Chrompack WCOT fused silica capillary column (50 m × 0.32 mm id) coated with CPWAX 57CB (1.13 µm coating); Chrompack, London) which terminated in the ion source of a Finnigan 4000 mass spectrometer. Helium was the carrier gas at 1 ml min<sup>-1</sup>. Volatile components were desorbed from the Tenax traps directly on to the capillary column by heating the trap at 250°C in the Unijector for 5 min while cooling the front of the column in dry ice/acetone. After desorption, the temperature was raised to 60°C and then at 4°C min<sup>-1</sup> to 230°C and held there for 10 min. The operating conditions of the mass spectrometer and the data collection system were the same as reported previously (Whitfield *et al* 1988). Retention times (RT) of compounds on the chromatogram were noted, and linear retention indices (LRI) for each compound were calculated by reference to the retention times for alkane standards (C<sub>9</sub>–C<sub>20</sub>) using the following formula.

$$\text{LRI} = \left( \frac{(\text{RT}_{\text{unknown}} - \text{RT}_{\text{alkane}})}{(\text{RT}_{\text{alkane}+1} - \text{RT}_{\text{alkane}})} + n_{\text{alkane}} \right) \times 100$$

Data were quantified by integration of the reconstructed ion chromatogram. To ensure that desorption of volatiles from each trap was comparable, 1,2-dichlorobenzene (50 ng) was added as an internal standard. The areas of the integrated 1,2-dichlorobenzene peaks were compared for the replicates and good agreement was found in all cases.

Pentan-1-ol and 2-pentylfuran were not sufficiently resolved on the chromatogram to be quantified by integration. The relative amounts of each component in the peak were estimated by studying individual scans within the peak and calculating the intensities of the ions at *m/z* 70 and *m/z* 81. Previous GC-MS runs had established that the total ion current for pentan-1-ol and 2-pentylfuran was proportional to the ion intensity at *m/z* 70 and *m/z* 81 multiplied by 5.22 and 1.89 respectively.

### GC eluate sniffing

A Carlo Erba Mega gas chromatograph equipped with a Unijector, operating in the concentrator/headspace mode, was fitted with the same CP52 column used for GC-MS. Samples were desorbed for 10 min at 230°C with the column cooled to 0°C by a

liquid CO<sub>2</sub> Carlo Erba cryocooling unit. After desorption, volatiles were chromatographed using the same temperature programme as for GC-MS. The column effluent was split in roughly equal proportions, and one part was routed through the FID detector to give a trace and the other part led to a sniffing port (Salter *et al* 1988). The odour of the eluate was judged by an assessor who noted the time, intensity and description of the odour. Alkane standards (C<sub>9</sub>-C<sub>20</sub>) were run separately so that LRI values could be calculated. Results from three assessors were collated and ranked on a scale of 1 to 6. Odours which were noted at the same retention time by the three assessors scored 3 points, and if the three descriptions agreed a further 3 points were awarded making the maximum of 6. For odours noted by two observers with no matching descriptions the score was 2 and so on. Only observations with a score of four or more were recorded in the collation.

### Preparation of acetals

Pentanal, hexanal or heptanal (Aldrich; 25 mg) were dissolved in petroleum ether (BP 40-60°C, 7 ml) and 3 ml of methanolic HCl was added with 5 ml of 1,1-dimethoxypropane (Supelco, Sawbridgeworth). Two layers formed, and after shaking the reaction was left overnight at room temperature (Mason and Waller 1964). The solutions were neutralised and dried by addition of Neutraid (NaHCO<sub>3</sub>: Na<sub>2</sub>CO<sub>3</sub>:Na<sub>2</sub>SO<sub>4</sub>, 2:1:2; oven dried at 110°C) and after 30 min an aliquot was taken for GC-MS analysis.

## RESULTS AND DISCUSSION

### Identification of volatiles

Paper extraction thimbles were used as supports during autoxidation to allow free access of air to the methyl arachidonate. Since small amounts (50 mg) were autoxidised, it was difficult to obtain an even spread of material on the thimble, and by dissolving the methyl arachidonate in ether, applying 1 ml to the thimble and removing the ether under nitrogen a more even distribution was achieved. With hindsight the use of an alternative solvent that does not form peroxides might have been advisable. In addition it was found that the extraction thimbles contained traces of copper and iron. Although the levels were not measured, information from Whatman suggested that 3 µg g<sup>-1</sup> Cu and 10 µg g<sup>-1</sup> Fe might have been present. This could have affected the autoxidation because of the known catalytic effects of these metals.

The volatile compounds isolated from methyl arachidonate oxidised alone or in a water emulsion are listed in Table 1. For each system, only compounds that were found in all three replicates were included. Identifications were made by comparison with mass spectra and linear retention indices of authentic compounds (MS, LRI) or by deduction from the mass spectrum alone (ms). The latter identifications must be regarded as tentative. Although quantitatively 12 volatiles accounted for 90% of the total, over 40 others were detected in trace amounts.

Autoxidation of methyl arachidonate has been shown to give six hydroperoxides of which 5-hydroperoxyarachidonate (18.3-36%) and 15-hydroperoxy-

TABLE 1  
Identity of volatiles from methyl arachidonate autoxidised with and without water

| Compound                             | Relative amount <sup>a</sup> |          | LRI  | Identity <sup>b</sup> |
|--------------------------------------|------------------------------|----------|------|-----------------------|
|                                      | Alone                        | Emulsion |      |                       |
| 1. Pentanal                          | ***                          | ***      | 977  | MS LRI                |
| 2. Decane                            | ***                          | ***      | 1000 | MS LRI                |
| 3. A methoxyhexene                   | **                           | *        | 1005 | ms                    |
| 4. 3-Methylpentan-2-one              | *                            | *        | 1018 | ms                    |
| 5. A methoxyhexene                   | **                           | **       | 1026 | ms                    |
| 6. Toluene                           | *                            |          | 1042 | MS                    |
| 7. A branched hydrocarbon            | *                            |          | 1048 | ms                    |
| 8. 1,1-Dimethoxypentane              | *                            | *        | 1065 | MS                    |
| 9. Hexanal                           | ***                          | ***      | 1083 | MS LRI                |
| 10. Butan-1-ol                       | *                            | *        | 1125 | MS LRI                |
| 11. 2-Butylfuran                     | *                            | *        | 1134 | MS                    |
| 12. Ethylbenzene                     | *                            | *        | 1134 | MS                    |
| 13. 1,4-Dimethylbenzene              | *                            | *        | 1142 | MS                    |
| 14. 1,3-Dimethylbenzene              | **                           | *        | 1148 | MS                    |
| 15. 1,1-Dimethoxyhexane              | ***                          | ***      | 1166 | MS LRI                |
| 16. Heptan-2-one                     | *                            | *        | 1182 | MS LRI                |
| 17. Methyl hexanoate                 | ***                          | **       | 1187 | MS LRI                |
| 18. 1,2-Dimethylbenzene              | *                            | *        | 1192 | MS                    |
| 19. Hex-2-enal                       | **                           | *        | 1219 | MS LRI                |
| 20. Pentan-1-ol                      | **                           | ***      | 1230 | MS LRI                |
| 21. 2-Pentylfuran                    | ***                          | *        | 1233 | MS LRI                |
| 22. Octan-3-one                      | *                            | *        | 1256 | MS LRI                |
| 23. Ethenylbenzene                   | **                           | *        | 1259 | MS                    |
| 24. 1,1-Dimethoxyheptane             | **                           | **       | 1266 | MS LRI                |
| 25. A 2-ketone                       | **                           | *        | 1270 | ms                    |
| 26. Pent-4-en-1-ol                   |                              | *        | 1280 | MS                    |
| 27. Methyl heptanoate                | *                            |          | 1287 | MS LRI                |
| 28. A trimethylbenzene               | **                           | *        | 1291 | ms                    |
| 29. Oct-1-en-3-one                   | ***                          | ***      | 1302 | MS LRI                |
| 30. An alkylbenzene                  | *                            |          | 1313 | ms                    |
| 31. Octan-2,3-dione                  | **                           | **       | 1318 | ms                    |
| 32. Hept-2-enal                      | ***                          | ***      | 1328 | MS LRI                |
| 33. A methyl heptenoate              | *                            | *        | 1346 | ms                    |
| 34. An alkylbenzene                  | *                            |          | 1348 | ms                    |
| 35. A methyl heptenoate              | *                            | *        | 1358 | ms                    |
| 36. Hex-2-en-1-ol                    | **                           | **       | 1391 | ms                    |
| 37. Nonanal                          | *                            | *        | 1396 | MS LRI                |
| 38. Oct-2-enal                       | **                           | ***      | 1404 | MS LRI                |
| 39. Oct-3-en-2-one                   | **                           | **       | 1408 | MS                    |
| 40. Acetic acid                      | **                           | *        | 1417 | MS LRI                |
| 41. Oct-1-en-3-ol                    | ***                          | ***      | 1434 | MS LRI                |
| 42. 2-Ethylhexan-1-ol                | *                            | *        | 1473 | MS                    |
| 43. 1,2-Dichlorobenzene <sup>c</sup> | **                           | **       | 1497 | MS LRI                |
| 44. A dimethoxy compound             | **                           | **       | 1504 | ms                    |
| 45. Benzaldehyde                     | *                            | *        | 1526 | MS LRI                |
| 46. Unknown MW 166                   | **                           | **       | 1536 | ms                    |
| 47. Octan-1-ol                       | *                            | *        | 1541 | MS LRI                |
| 48. Unknown (isomer of 47)           | *                            | *        | 1553 | ms                    |

(continued)

TABLE 1—*contd.*

| Compound                        | Relative amount <sup>a</sup> |          | LRI  | Identity <sup>b</sup> |
|---------------------------------|------------------------------|----------|------|-----------------------|
|                                 | Alone                        | Emulsion |      |                       |
| 49. Octa-2,4-dienal             | *                            | *        | 1591 | MS                    |
| 50. Oct-2-en-1-ol               |                              | *        | 1594 | ms                    |
| 51. Nonanol                     | *                            | *        | 1643 | MS LRI                |
| 52. Dec-2-enal                  |                              | *        | 1649 | MS                    |
| 53. Acetophenone                | **                           |          | 1655 | MS                    |
| 54. A pentene ether compound    |                              | **       | 1656 | ms                    |
| 55. Pentanoic acid              | *                            | *        | 1706 | MS LRI                |
| 56. Methyl 4(2-furyl) butanoate | *                            | *        | 1743 | MS                    |
| 57. Naphthalene                 | *                            |          | 1757 | MS                    |
| 58. An unsaturated alcohol      | **                           |          | 1767 | ms                    |
| 59. Deca-2,4-dienal             | *                            | *        | 1800 | MS LRI                |
| 60. Hexanoic acid               | **                           | **       | 1812 | MS LRI                |
| 61. Deca-2,4-dienal             | *                            | *        | 1813 | MS LRI                |

<sup>a</sup> Relative amount calculated from integrated chromatogram. \*\*\* > 100 000, \*\* > 10 000, \* > 1000 (ion current; arbitrary units).

<sup>b</sup> Methods of identification: MS LRI, Mass spectrum and LRI agree with those of authentic compound; MS, mass spectrum agrees with literature spectrum (Heller and Milne 1978); ms, interpretation of mass spectrum.

<sup>c</sup> Internal standard.

arachidonate (33–33.8%) predominate (Terao and Matsushita 1981; Yamagata *et al* 1983). Significant amounts of the 8,9,11- and 12-hydroperoxy derivatives are also formed (9.4–15.6%). When methyl arachidonate or arachidonic acid were oxidised in an emulsion system catalysed by haemprotein, results were similar although the proportions in which the isomers were found differed slightly: 5- and 15- derivatives 24–31%, 9- and 11- derivatives 12–16%, 8- and 12- derivatives 5–10% (Terao and Matsushita 1981). Differences in hydroperoxides were noted when singlet oxygen was the oxidant (eight hydroperoxides formed) or when tocopherol was present (equal amounts of all six formed) (Terao and Matsushita 1981; Yamagata *et al* 1983; Frankel 1985).

The hydroperoxides decompose further and the compounds expected from 15-hydroperoxyarachidonate cleaving from the alkyl end of the chain (Grosch 1982) are hexanal, pentane, pentanal and pentan-1-ol. All except pentane (which is too volatile to be detected on the headspace system) were identified as major volatiles in autoxidised methyl arachidonate (Table 1). Compounds from the 12-hydroperoxy derivative were also found, namely oct-1-en-3-one and oct-1-en-3-ol. 2-Pentylfuran is a product of 11-hydroperoxyarachidonate decomposition but there was only a small amount of deca-2,4-dienal also expected from this hydroperoxide and found in significant quantities by other workers (Badings 1970). Experiments showed that the headspace system was capable of trapping deca-2,4-dienal at the collection temperatures used (20 °C) despite its relative low volatility. Similarly, dienals were found only in the emulsion system as minor components. The low levels of dienals

may be explained by the different methods used for autoxidation. Badings (1970) used arachidonic acid rather than the methyl ester, and also used 1 g of the compound in a round-bottomed flask. Thus access of oxygen was somewhat limited and the solubility of deca-2,4-dienal in fat may have allowed it to accumulate. In the thimble system used in this study, methyl arachidonate was finely distributed, which permitted ready uptake of oxygen and further reaction of the dienals. This is supported by the work of Michalski and Hammond (1972) who found that deca-2,4-dienal was readily oxidised at room temperature to the corresponding acid together with non-2-enal, oct-2-enal and heptanal. Deca-2,4-dienoic acid would not be sufficiently volatile to be extracted under the conditions used in this study but the presence of substantial quantities of hept-2-enal and smaller quantities of oct-2-enal may be due to further oxidation of dienals. In the autoxidation of methyl linoleate on a glass wool matrix (Horvat *et al* 1965) no dienals were found although previous studies had identified both nona-2,4-dienal and deca-2,4-dienal as major products. This supports the view of Grosch (1987) that, under conditions where oxygen is readily available, further oxidation of dienals occurs.

The other major volatiles, 1,1-dimethoxyhexane, methyl hexanoate and hexanoic acid, were not expected from the initial decomposition of hydroperoxides. However, they could have been formed from hexanal:hexanoic acid by oxidation and the other two compounds by reaction of methanol with hexanal and hexanoic acid. 1,1-Dimethoxyhexane and methyl hexanoate have also been reported in the volatiles from autoxidised linoleate (Horvat *et al* 1965). 1,1-Dimethoxyhexane as well as its heptane and octane homologues (LRI 1065, 1166, 1266) were positively identified by comparison with mass spectra and LRI values of the authentic compounds synthesised from the corresponding aldehydes.

Several alkylbenzenes were identified in the headspace of both systems (Table 1), and although they are often found as artefacts (from solvent residues or from Tenax degradation), control experiments with no methyl arachidonate showed only traces of a few alkylbenzenes. Alkylbenzenes are known oxidation products of lipids and are found in most heated foods (Forss 1972). Possible routes to the aromatic compounds include the oxidation of unsaturated hydrocarbons (Min *et al* 1977) or alka-2,4-dienals (Nonaka *et al* 1967), both products of the autoxidation of fatty acids.

The compound at LRI 1743 was tentatively identified as methyl 4(2-furyl)butanoate from the mass spectrum which contained six significant ions [94(100%), 81(42%), 53(27%), 43(10%), 168(7%), 137(4%)]. The ion at  $m/z$  168 corresponds to the molecular weight of the compound while the base peak at  $m/z$  94 can be explained by McLafferty rearrangement of the methyl ester part of the molecule leading to loss of 74 mass units. Other ions at  $m/z$  137 (loss of methyl) and  $m/z$  81 (typical of alkylfurans) support the proposed structure.

Other compounds could not be identified although some structural features were apparent. Table 1 shows the 10 most abundant ions for these compounds. The peaks at LRI 1536 and 1553 gave mass spectra which were practically identical suggesting that two isomers were present. The spectra suggested alkylfurans or dienals, but comparison of LRI values of known compounds did not agree with the apparent molecular ion at  $m/z$  166. The compounds may be alkylpyrans although

no standards were available for comparison. When  $\text{H}_2\text{S}$  was reacted with deca-2,4-dienal (Mottram D J, Salter L J unpublished results), a series of alkylthiophenes was found together with isomeric compounds that were tentatively identified as 2-alkyl-2*H* thiapyrans. Alkylpyrans may be produced in an analogous way by the reaction of water with dienals.

### Quantification

Identification of the volatiles from autoxidation of methyl arachidonate with and without water showed little difference in the type of compounds formed, although the proportions appeared to vary (Table 1). In order to quantify this variation, initial experiments were performed to measure the inherent variation during the autoxidation of methyl arachidonate. Table 2 shows the relative amounts of the 12 major volatiles in two experiments (A and B) carried out on different days. Conditions for the autoxidations were maintained as similar as possible. The amounts of volatiles formed were calculated following integration of the reconstructed ion chromatogram. Because of the small amount of data available the results of any statistical evaluation need to be treated with caution. To give some indication of variation between the three replicates, the percentage coefficients of variation ( $100 \times \text{standard deviation/mean}$ ) for the 12 compounds in Table 2 were calculated. The range was 3.2–85.7% with a mean of 23.1%. When the data in experiments A and B were compared using the Student's *t*-test, all the components except pentanal showed significant differences between experiments. Thus there was considerable variation between replicates and even more variation between

**TABLE 2**  
Composition of major volatiles from autoxidised methyl arachidonate

| Compound            | Treatment  |            |               |
|---------------------|------------|------------|---------------|
|                     | Alone<br>A | Alone<br>B | Emulsion<br>C |
| Pentanal            | 4.41       | 4.86       | 6.23          |
| Decane              | 0.28       | 3.19       | 0.24          |
| Hexanal             | 6.22       | 17.41      | 11.82         |
| 1,1-Dimethoxyhexane | 2.56       | 9.50       | 1.71          |
| Methyl hexanoate    | 0.21       | 2.08       | 0.26          |
| Pentan-1-ol         | 0.87       | 1.22       | 1.61          |
| 2-Pentylfuran       | 0.67       | 1.40       | 0.60          |
| Oct-1-en-3-one      | 9.49       | 5.22       | 7.88          |
| Hept-2-enal         | 16.45      | 10.53      | 11.08         |
| Oct-2-enal          | 3.24       | 0.62       | 2.15          |
| Oct-1-en-3-ol       | 39.46      | 32.91      | 46.13         |
| Hexanoic acid       | 5.95       | 1.30       | 1.59          |

The mean values for each treatment are calculated from three replicates and are expressed as a percentage of the total ion current from the GC-MS data.

**TABLE 3**  
Odour of volatiles from methyl arachidonate autoxidised alone and in an emulsion

| <i>LRI</i>   |                 | <i>Description</i>                      |
|--------------|-----------------|---|
| <i>Alone</i> | <i>Emulsion</i> |   |
| 978          |                 | Green aldehyde/green aldehyde           |
|              | 979             | Sharp aldehyde/cheesy/smoky             |
| 1046         |                 | Burnt/metallic rubbery/rubbery          |
|              | 1048            | Burnt rubber/burnt paper/sharp rubbery  |
| 1085         |                 | Green sweet/green aldehyde/green leaves |
|              | 1086            | Sweet green/green grassy/green old      |
|              | 1115            | Fruity green/green solvent              |
|              | 1123            | Fruity metallic/solvent green/sickly    |
| 1124         |                 | Metallic acrid/metallic/sweet green     |
| 1137         |                 | Medicine/aromatic                       |
|              | 1228            | Green/green plastic                     |
|              | 1241            | Polish/aldehyde/plastic                 |
|              | 1300            | Green/floral                            |
| 1308         |                 | Grass/herbal fatty/old green            |
|              | 1310            | Grassy green/vegetation/green smoky     |
|              | 1335            | Flowery/warm mealy/floral sickly        |
| 1336         |                 | Almonds/waxy almonds/metallic old       |
| 1399         |                 | Soap/wax/soap perfume                   |
|              | 1403            | Green floral/perfume                    |
| 1405         |                 | Sweet green/sweet fruity                |
|              | 1411            | Pleasant lemon/green old/green citrus   |
| 1412         |                 | Grass/sweaty green/waxy                 |
|              | 1434            | Foul pungent/meally green/green pungent |
|              | 1445            | Grassy green/mealy/pungent green        |
| 1461         |                 | Wax/laurel leaves hedge                 |
|              | 1497            | Floral vanilla/fruity melon             |
|              | 1600            | Lemon metallic/waxy painty/lemon green  |
| 1603         |                 | Aldehyde/waxy aldehyde                  |
|              | 1646            | Onions/unpleasant sweaty/sweetish       |
|              | 1671            | Metallic/mealy/greenish                 |
|              | 1712            | Green waxy/green earthy/green PVC       |
|              | 1763            | Tropical green/woody mealy/earthy       |
|              | 1778            | Green waxy/green floral                 |
| 1822         |                 | Paint wax/mealy/plastic green           |
|              | 1823            | Green waxy/hot fatty/acrid floral       |
| 1863         |                 | Burnt food/burnt                        |
|              | 1863            | Smoked food/cooked food                 |
|              | 1947            | Green herbal/sweet floral               |
| 1987         |                 | Sweaty mealy/waxy candles               |
|              | 1988            | Unpleasant sweaty/earthy smoky          |
| 2012         |                 | Old burnt paint/paint                   |

experiments. Whether this was due to differences in the extent of oxidation of the starting material or due to variation in environmental conditions during autoxidation was not clear. Gas chromatography of methyl arachidonate demonstrated its high purity (99%), but UV spectrophotometry of a dilute solution ( $0.5 \text{ mg ml}^{-1}$ ) showed significant absorption (0.29 AU at 232 nm). Bergstrom (1945) suggested that absorption in this region was indicative of conjugated dienes and therefore could be used as a measure of autoxidation. The differences between experiments conducted on different days could therefore be attributable to the different extent of oxidation of the starting material. The difficulties of purifying micro amounts of methyl arachidonate and preventing further oxidation after purification were such that the material was used without further treatment. Since the replicates within each experiment agreed within the limits (mean variation about 20%) expected for reactions of this nature, it was considered adequate to carry out the comparisons described below using methyl arachidonate from the same batch and at the same time.

To study the effect of water on the amounts of individual volatiles formed during autoxidation, two sets of flasks containing either methyl arachidonate (B) or an emulsion of methyl arachidonate (C) was set up and autoxidised at the same time. Analysis of the volatiles by GC-MS and quantification of the major peaks gave the results in Table 2. Four components, hexanal, pentan-1-ol, hept-2-enal and hexanoic acid, were present in the same proportions in both treatments. Two components, 1,1-dimethoxyhexane and methyl hexanoate, were present at significantly lower levels in the emulsion system. These two compounds are not stable in aqueous acidic media, which might explain the reduced amounts. Overall, however, water had little effect on the type or amounts of volatiles formed although in the trace components increased amounts of alcohols were formed (Table 1).

#### **Odour of components in autoxidised methyl arachidonate**

The results of the odour assessments are shown in Table 3. While some odours can be associated with identified peaks in the trace obtained from the FID detector, others do not coincide with a particular peak or are associated with compounds that were not present in sufficient quantities for identification. For the autoxidation of methyl arachidonate, 16 odours were awarded scores of 4 or more out of a total of 79 noted odours. Green, waxy and metallic were the descriptors used most frequently, and these describe the overall odour of autoxidised methyl arachidonate. For the emulsion system, 25 odours scored 4 or more out of a total of 100 odours. The overall smell was similar, probably due to the high concentration of oct-1-en-3-ol masking other odours. In neither sample was there a single component that had the characteristic overall odour.

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## REFERENCES

- Badings H T 1970 Cold storage defects in butter and their relation to the autoxidation of unsaturated fatty acids. *Neth Milk Dairy J* **24** 146–256.
- Bergstrom S 1945 Autoxidation of linoleic acid. *Nature* **156** 717–718.
- Ellis R, Gaddis A M, Currie G T 1966 Carbonyls in oxidizing fat. IX. Aldehydes isolated from methyl arachidonate. *J Food Sci* **31** 191–195.
- Forss D A 1972 Odor and flavor compounds from lipids. In: *Progress in the Chemistry of Fats and Other Lipids XIII*, ed Holman R T. Pergamon Press, New York. Part 4.
- Frankel E N 1985 Chemistry of free radical and singlet oxidation of lipids. *Progr Lipid Res* **23**(3) 197–221.
- Grosch W 1982 Lipid degradation products and flavour. *Food Flavours, Part A*, eds Morton I D & Macleod A J. Elsevier, Amsterdam, pp 325–398.
- Grosch W 1987 Reactions of hydroperoxides—products of low molecular weight. *Autoxidation of Unsaturated Lipids*, ed Chan H W-S. Academic Press, London, pp 95–139.
- Heller S R, Milne G W A 1978 *EPA/NIH Mass Spectral Data Base*. National Bureau of Standards, Washington, DC (and supplements 1980, 1982).
- Horvat R J, McFadden W H, Ng H, Black D R, Lane W G, Teeter R M 1965 Volatile products from mild oxidation of methyl linoleate. Analysis by combined mass spectrometry–gas chromatography. *J Amer Oil Chem Soc* **42** 1112–1115.
- Labuza T P 1971 Kinetics of lipid oxidation in foods. *Crit Rev Food Technol* **2** 355–405.
- Mason M E, Waller G R 1964 Dimethoxypropane induced transesterification of fats and oils in preparation of methyl esters for gas chromatographic analysis. *Anal Chem* **36** 583–586.
- Michalski W T, Hammond E G 1972 Use of labelled compounds to study the mechanism of flavor formation in oxidizing fats. *J Amer Oil Chem Soc* **49** 563–566.
- Min D B S, Ina K, Peterson R J, Chang S S 1977 The alkylbenzenes in roast beef. *J Food Sci* **42** 503–505.
- Nonaka M, Black D R, Pippen E L 1967 Gas chromatographic and mass spectral analyses of cooked chicken meat volatiles. *J Agric Food Chem* **15** 713–717.
- Salter L J, Mottram D S, Whitfield F B 1988 Volatile compounds produced in Maillard reactions involving glycine, ribose and phospholipid. *J Sci Food Agric* **46** 227–242.
- Terao J, Matsushita S 1981 Analysis of hemoprotein catalysed peroxidation products of arachidonic acid by GC–MS. *Agric Biol Chem* **45** 595–599.
- Whitfield F B, Mottram D S, Brock S, Puckey D J, Slater L J 1988 The effect of phospholipid on the formation of volatile heterocyclic compounds in heated aqueous solutions of amino acids and ribose. *J Sci Food Agric* **42** 261–272.
- Yamagata S, Murakami H, Terao J, Matsushita S 1983 Nonenzymatic oxidation products of methyl arachidonate. *Agric Biol Chem* **47** 2791–2799.



## Acid Hydrolysis Prior to Automatic Analysis for Starch

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**Key words:** Potatoes, starch, determination, acid hydrolysis, enzymic hydrolysis.

*The determination of starch in potatoes (*Solanum tuberosum* L) by enzymic hydrolysis with amyloglucosidase (Hudson et al 1976), compared with the acid hydrolysis procedure suggested here, has several disadvantages. The extraction and hydrolysis stage is slower (21 h cf 5 h per batch of 30), activity of the enzyme may vary, reagents are more expensive, and complete hydrolysis is more difficult. Its main advantage is that it is more specific, not hydrolysing the non-cellulosic polysaccharides of the cell walls. This communication examines whether an acid hydrolysis method is an acceptable, even preferable, method for determining starch in freeze dried potato samples. Effects of drying, hydrolysis and associated errors are quantified.*

The sample is milled to 1 mm, and 100.0 mg is weighed into a beaker and is extracted with 10 ml aqueous ethanol (100 ml C<sub>2</sub>H<sub>5</sub>OH litre<sup>-1</sup>) for 30 min to dissolve sugars, dextrans and tannins, which amount to about 120 mg g<sup>-1</sup> DM. As filtration results in a ~6% loss of starch by adherence to the paper, the suspension is centrifuged at 1500 × *g* for 5 min and the residue is washed into McCartney bottles, graduated at 15 ml, with 1 M HCl, with which it is made up to the mark. The cap, fitted with a PTFE liner, is screwed on firmly prior to heating at 106 °C for 40 min. (Glucose production on hydrolysis was 2 mg after 10 min, 94 mg after 20, 40 and 60 min.)

After cooling, the contents are washed into 150-ml beakers containing 50 ml H<sub>2</sub>O, adjusted to pH 3.0 ± 0.2 and made up to 100 ml. After settlement, 10-ml aliquots are made up to 50 ml with saturated benzoic acid solution resulting in a 5000-fold dilution of the original samples. Fructose standards in saturated benzoic acid are used in the Technicon AutoAnalyzer method described by Thomas (1977). Standard BDH potato starch, and low and high starch content potato controls, should be included.

The precision obtained from ten replicates of standard potato starch (15.9% H<sub>2</sub>O) with each solution analysed in triplicate was a standard deviation of ± 0.7 at a level of 96 mg glucose. Recovery of added starch obtained from five replicates

analysed in triplicate was 102%, which is almost within experimental error of  $\pm 1.5\%$  of 100%. A potato sample bulked from mixed cultivars (8.6%  $\text{H}_2\text{O}$ ),  $5 \times 3$  replicates, gave  $s = \pm 1.4$  at a level of 76 mg glucose.

Dry potato starch shows a great affinity for water and may regain over half its weight at relative humidities over 90% (Schierbaum 1960). A 33% regain corresponds to  $3\text{H}_2\text{O}$ , and 44% to  $4\text{H}_2\text{O}$  per residue (Blackwell *et al* 1969). An earlier empirical formula for air-dry starch was  $\text{C}_{36}\text{H}_{62}\text{O}_{31} \cdot 12\text{H}_2\text{O}$  ( $2.17\text{H}_2\text{O}$  per residue of  $\text{C}_6\text{H}_{10}\text{O}_5$ ), the  $12\text{H}_2\text{O}$  being susceptible to removal by drying at 100–110°C (Sachsse 1877). Drying at 90°C removed 15.9% by weight, or  $1.7\text{H}_2\text{O}$  per residue, and freeze drying 12% by weight, or  $1.2\text{H}_2\text{O}$  per residue. Theoretical and observed levels of glucose production (mg) from a 100 mg sample are as follows:

|   |       |
|---|-------|
| Undried standard potato starch  | 89.6  |
| Freeze dried  | 101.7 |
| Oven dried at 90°C  | 106.6 |
| Oven dried at 105°C   | 109.1 |
| From $\text{C}_6\text{H}_{10}\text{O}_5$ to $\text{C}_6\text{H}_{12}\text{O}_6$ | 111.1 |
| Observed value corrected for $\text{H}_2\text{O}$                               | 124.0 |

Because the observed value (124.0) is 11.6% higher than the theoretical maximum (111.1), it is postulated that hydrolysis also degraded glucose to give products exerting a greater chromogenic effect on anthrone than glucose itself. This was proved by subjecting 100 mg pure glucose to hydrolysis for  $\frac{1}{2}$ , 1 and  $1\frac{1}{2}$  h at 106°C. The similar values averaged 113 mg 'glucose' using colorimetry. Accordingly, 100 mg starch would have given the equivalent value of:

$$\begin{aligned} (180.16/162.14) \times 113 &= 125.6 \text{ for starch as } \text{C}_6\text{H}_{10}\text{O}_5 \\ (1080.96/990.86) \times 113 &= 123.3 \text{ for starch as } \text{C}_{36}\text{H}_{62}\text{O}_{31} \end{aligned}$$

These values are close to the observed value of 124.0. It is known that *D*-fructose is the only isomer formed from the action of 0.45 M sulphuric acid on *D*-glucose at 120°C, albeit in small amounts (Ohno and Ward 1961). Hydrochloric acid (1 M) is about twice as vigorous, and it would not be surprising if larger quantities of fructose were formed. Fructose standard solutions were found to give readings 14% higher than equivalent glucose ones. This would explain the elevated observed value.

It is also well known that *D*-glucose is dehydrated in the presence of acids to produce mainly 5-hydroxymethylfurfural (HMF) amounting to less than 1% under usual starch hydrolysis conditions (BeMiller 1965). Laevulinic and formic acids are also formed. Assuming complete breakdown of glucose to each of these substances, the observed peak heights from their equivalent solutions corresponded to the following amounts of glucose: HMF 140, laevulinic acid 0.3, and formic acid 0.9 mg. Thus HMF exhibits a greater chromogenic effect with anthrone than either glucose or fructose. It is reported that the anthrone colorimetric reaction is based on the conversion of soluble carbohydrates to HMF by hot  $\text{H}_2\text{SO}_4$ , with subsequent reaction with anthrone and resin products to give the colour (Hoermann and Siddiqui 1968). This probably does not go to completion in practice, otherwise glucose and fructose would give the same peak heights. Oven dried (105°C) starch

should produce 109.1 mg glucose. If this were all converted to fructose, the expected peak height would correspond to:

$$109.1 \times 114/110 = 124.4 \text{ mg glucose}$$

This is within experimental error ( $\pm 1.8$ ) of the observed value of 124.0. The actual quantities of fructose and/or HMF could be determined by HPLC (Binder 1980) with identification confirmation by IR but such facilities are unavailable in this laboratory. However, an IR scan of an ethanolic extract of starch hydrolysate revealed a peak at  $1650 \text{ cm}^{-1}$  typical of the C=C of HMF.

Calculation of results when using fructose standards is achieved using the formula:

$$\% \text{ Starch} = (F/200) \times 100/(100 - W) \times 0.9 \times 100\%$$

where  $F$  = fructose ( $\text{mg litre}^{-1}$ ),  $W$  = % sample moisture from separate determination, and  $0.9$  = correction for conversion of  $\text{C}_6\text{H}_{12}\text{O}_6$  to  $\text{C}_6\text{H}_{10}\text{O}_5$ . If glucose standards are used, multiplication by  $0.8$  corrects for fructose/HMF formation.

Some foodstuffs might only rehydrate with difficulty. However, freeze dried potato powder showed no adverse behaviour. The possible hydrolysis of cell walls to give interfering substances resulting in an over-correction of starch was investigated. Six 1.000-g replicates of two samples of potato were subjected to the VanSoest neutral detergent procedure, yielding 62 and 55 g cell walls  $\text{kg}^{-1}$ . Replicates were combined and, after acid hydrolysis and colorimetry, produced equivalents of 2.0 and 1.8 mg glucose from a 1.0-g sample. Thus starch would overestimate by about 2%, which should be corrected.

It is concluded that the above acid hydrolysis procedure is rapid, reliable, precise (2 s (95% confidence limit) =  $\pm 1.4$  at a level of 96 mg glucose production) and sufficiently accurate to be used instead of enzymic hydrolysis for the routine analysis of starch in potatoes.

## REFERENCES

- BeMiller J N 1965 Acid hydrolysis and other lytic reactions of starch. In: *Starch: Chemistry and Technology*, eds Whistler R L & Paschall E F. Academic Press, New York, p 513.
- Binder H 1980 Separation of monosaccharides by high-performance liquid chromatography: comparison of ultraviolet and refractive index detection. *J Chromatogr* **189** 414–420.
- Blackwell J, Sarko A, Marchessault R H 1969 Chain conformation in  $\beta$ -amylose. *J Mol Biol* **42** 379–383.
- Hoermann H, Siddiqui I A 1968 Color reactions of carbohydrates. IV. Products of the color reaction of *D*-fructose and *D*-glucose with anthrone sulphuric acid. *Justus Liebig's Ann Chem* **714** 174–190.
- Hudson J H, John P M V, Bailey B S, Southgate D A T 1976 The automated determination of carbohydrates and its application to foodstuffs. *J Sci Food Agric* **27** 681–687.
- Ohno Y, Ward K J 1961 Acid epimerisation of *D*-glucose. *Org Chem* **26** 3928–3931.
- Sachse R 1887 Über die Stärkeformel und über Stärkebestimmungen. *Chem. Zentralbl* 732–736.
- Schierbaum F 1960 Die Hydratation der Stärke. *Die Stärke* **12** 237–243.
- Thomas T 1977 An automated procedure for the determination of soluble carbohydrates in herbage. *J Sci Food Agric* **28** 639–642.



## **Agriculture Group Symposium Hydroponics Update**

*The following are summaries of papers presented at a joint meeting of the Agriculture Group of the Society of Chemical Industry and the British Society of Soil Science held on 31 May 1989 at the Pershore College of Horticulture, Worcestershire. The papers published here are entirely the responsibility of the authors and do not reflect the views of the Editorial Board of the Journal of the Science of Food and Agriculture.*

### **Use of Sodium Chloride for Tomatoes Grown in Solution Culture**

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Tomato is classified by Lunt (1966) as a plant which shows a growth response to sodium only where the supply of potassium is insufficient. With soil-grown glasshouse crops care is taken to minimise sodium chloride levels because of possible adverse effects of sodium upon soil structure and the association of chloride with unwanted salinity. However, recent experience has indicated that tomatoes grown in solution culture can tolerate higher levels of sodium chloride than was previously considered desirable for soil-grown crops. Several workers have successfully used sodium chloride to increase the conductivity of the nutrient solution in nutrient film culture, and have obtained improvements in fruit quality. The effect upon yield is less certain, with Adams (1988) reporting a small increase (6%) and van der Burg *et al* (1986, 1987) a small decrease (3–5%) from increasing conductivity to a level above that normally used in commercial practice.

The experiments referred to above used sodium chloride to increase conductivity thereby confounding conductivity effects with any specific sodium chloride effects. A different approach was followed in an experiment with tomatoes grown in rockwool at ADAS, Wye. Treatments in which sodium chloride partially replaced calcium and potassium nitrates were compared with the basic nutrient solution. All treatments were maintained at the same conductivity level. Over a 16-week cropping period the sodium chloride treatments reduced yield by an average of 6%. Reasons for this are unclear as leaf analysis showed adequate nutrient levels. Tomatoes grown with sodium chloride were preferred by taste panels because of an enhanced perception of saltiness. This was associated with higher levels of sodium

and chloride in the fruits, with little difference in dry matter, sugar or acidity levels between treatments. In contrast the effect of increased conductivity upon fruit quality is associated with reduced water uptake with a consequent increase in dry matter, sugar and acidity levels in the fruits (Hobson 1988).

Leaf magnesium concentration was increased by sodium chloride indicating that sodium is less antagonistic than potassium to magnesium uptake.

Sodium chloride offers a cheaper method of increasing conductivity for early growth control and improving fruit quality with little or no yield penalty. For rockwool systems where 15–20% of the nutrient solution is drained to waste it will reduce the potential for nitrate pollution. There are indications that sodium chloride can have specific effects upon fruit quality additional to those associated with increased conductivity levels.

### References

- Adams P 1988 Some responses of tomatoes grown in NFT to sodium chloride. *Proc 7th Int Soilless Culture*, Flevohof.
- Hobson G (1988) How the tomato lost its taste. *New Scientist* 29 September 46–50.
- Lunt O R 1966 Sodium. In: *Diagnostic Criteria for Plants and Soil*, Ed. Chapman H D. University of California, p 411.
- van der Burg A M M, Theune D, Sonneveld C 1986 Specific effects of sodium chloride on tomatoes grown in nutrient film. Glasshouse Crops Research Station. Naaldwijk. Annual Report 1985, pp 21–22.
- van der Burg A M M, Theune D, Sonneveld C 1987 Specific effects of sodium chloride on tomatoes grown in nutrient film. Glasshouse Crops Research Station. Naaldwijk. Annual Report 1986, p 16.

## Nutrient Film Culture (NFT)

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### *The basic system*

Nutrient film culture is a specialised form of solution culture ('hydroponics') in which the plants are grown in a very shallow stream of nutrient solution flowing past the roots. The main components of the system are a catchment tank containing a complete nutrient solution, a pump to deliver the solution, via pipes, to the upper ends of the gullies in which the crop is grown, and a return pipe to collect the solution flowing from the lower ends of the gullies and to discharge it into the catchment tank in a cascade, thus aerating it for recirculation.

### *Automated controls*

The water level may be maintained by a solenoid valve and level sensor or by a simple float-valve. Dilute nitric acid is added by a small pump when the pH of the

solution rises above a set point, eg pH 5.8. Concentrated nutrient solutions are injected into the tank by pumps whenever the electrical conductivity (ie the total salt content) of the recirculating solution declines below a predetermined value.

### *Commercial applications*

Successful cropping with NFT requires more skill and attention to detail than other soilless substrates. This is mainly due to the recirculation of the nutrient solution; there is no drainage or leaching. Nevertheless, some growers in the UK have been using the system for over 10 years, and 36 ha (about 90 acres) of early heated tomatoes were grown in England in 1988. The commercial potential of NFT for tomato production is considerable. For example, the highest yields in the Grower Recording Scheme in 1986 and 1987 were achieved in NFT, and the fruit quality was very good. Expansion of the largest NFT unit in the world (at Selby, Yorkshire) from 8 ha to 12 ha reflects remarkable confidence in the system.

### *Research applications*

Nutrient film culture has been used to obtain accurate assessments of the amounts of nutrients absorbed by plants. The uptakes of nitrogen and potassium by tomatoes are both closely related to water uptake, being influenced most by light intensity and air temperature. It was found that these rates were low at dawn, increased with the light intensity and generally reached a maximum during the brightest part of the day. Then the rates of uptake declined again and remained at a minimum during the night. In contrast, the uptake of phosphorus appears to be closely related to solution temperature and reaches a maximum rate somewhat later in the day than is found for nitrogen and potassium.

## **Environmental Control and Blossom End Rot**

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Blossom end rot (BER) is caused by a deficiency of calcium available to fruit as they expand. Causes include obvious damage to root systems or nutritional imbalance in feed solutions. Excessive conductivities in hydroponic systems, localised water shortages due to blocked irrigation nozzles, or waterlogging due to poor drainage will also cause problems. Certain environmental conditions can also cause BER, but because of the delay between the conditions occurring and the appearance of the symptoms they can often be difficult to identify.

In long-season tomato crops, BER does not generally become a problem until the spring. In two years' work at Efford EHS, early sown tomatoes were grown in a range of humidity conditions between January and early March. In 1987 continuous high humidity day and night for 4 weeks from mid January (up to 95 °

RH) gave severe calcium deficiency symptoms on the leaves and greatly reduced leaf area. In 1988 similar regimes were examined for 7 weeks from mid January but without the same severity of symptoms developing. In 1988 growing conditions were more favourable with increased transpiration due to solar radiation during the treatment period. In neither year was there any evidence of BER developing as a result of these treatments.

BER typically appears first in April or May. This is when plants frequently have maximum fruit load and minimum root activity. In spring-sown crops, over-vigorous vegetative growth can direct water away from fruit especially in dry conditions and BER can be severe on early trusses. There is evidence from tomato crops grown in warmer climates that temperature fluctuation can cause BER, particularly if cold nights are followed by warm days leading to water stress in the plants. It is known that high humidities at night lead to root pressure whereby transpiration through leaves is restricted but roots actively pump water and therefore calcium into tissues which transpire more slowly such as fruit. This is particularly applicable on nights when pipe heat is being used, which stimulates root activity. Such conditions may occur in the early months of a long-season crop and may help to explain why BER is not then a serious problem. Drier conditions occurring in the spring when more ventilation is being given during the day may draw water from the fruit during the day which the plants find difficult to replace at night by root pressure, particularly if root activity is reduced due to heavy fruit load or reduced pipe heat.

A recent BER problem at Efford EHS was traced back to a period in early April when temperatures dropped and light conditions were relatively poor. Night humidities in particular were reduced due to a considerable increase in the pipe heat needed to maintain temperature. This probably led to increased leaf transpiration at night and reduced root pressure leading to BER which was seen approximately three weeks later on developing fruit.

Clearly it is important to prevent the conditions likely to lead to BER. Maintaining uniform conditions of humidity and temperature would seem to be important but variations in ambient conditions make this difficult at times. Excessively high or low humidities during the day can both cause water stress, and at night low humidities are undesirable but high night humidities can lead to condensation on fruits as temperatures rise in the early morning which has implications for other aspects of fruit quality. The best that can be achieved is, as ever, likely to be a compromise.

## **Rockwool**

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Rockwool is an inert material which is used widely for insulation. It is made by melting rock, most commonly a form of basalt, and 'spinning' it into fibres.

Rockwool for horticultural use is treated to make it water absorbent for growing crops or water repellent for compost incorporation.

The system was developed initially in Denmark in the early 1970s and was quickly taken up by the Dutch (Smith 1987). The technique spread to England in the late 1970s, particularly to the Humberside area. Development work at Stockbridge House and Efford Experimental Horticulture Stations showed the benefits in yield and quality over peat- and soil-based systems.

In England and Wales in 1989 there will be approximately 159 ha of tomatoes, 172 ha of cucumbers and 7 ha of peppers grown on rockwool (Butters 1989). This represents about 48% of the heated tomato crop and 81% of the total cucumber area.

One of the essential features of the standard rockwool system is that modules are isolated from one another. Provided drainage is adequate, root diseases cannot spread from slab to slab.

The amount of feed applied varies with environmental conditions but plants may be watered two or three times an hour under maximum insolation. All excess feed normally runs to waste; the amount of waste depends on the variation between drip nozzles and the quality of the water. If the water contains high levels of sodium or chloride, these elements tend to build up in the slabs and must then be flushed out periodically. Cucumbers have been grown successfully with  $235 \text{ mg litre}^{-1}$  Na in the water supply; levels in the slab solution reached  $500 \text{ mg litre}^{-1}$  Na. For tomatoes, sodium chloride has been added to feeds to control growth and improve flavour without serious adverse effects (Marks 1989).

As with any other hydroponic system, regular monitoring is essential. Growers are recommended to check the pH and conductivity in the slabs every day and to have a full nutrient analysis every 2 weeks. The amount of run-off must also be measured, either with a home-made system or with various automated systems.

In 1988 a commercial crop of rockwool tomatoes was monitored to attempt a nutrient balance over a whole season. Apparent recovery of applied nitrogen, phosphorus and potassium was about 60%. Calcium, magnesium and most trace elements showed relatively low recovery rates of between 23 and 48%. Manganese was taken up more efficiently than any other nutrient (78%).

Once a grower has equipped to grow in an inert, free-draining substrate, materials other than rockwool can be used. There are several new growing media available at present, though as yet they represent a very small area. They include polyurethane foam and polyacrylamide gel.

Although rockwool slabs can be sterilised and re-used for at least one extra season, they must eventually be disposed of; this is a considerable problem and expense.

In the future, it may no longer be acceptable to allow excess nutrient solution to run to waste. Tomatoes grow very well in NFT, but so far long-season cucumber crops have been unsuccessful. Recirculating rockwool systems have been trialled at experimental centres in England and Holland. The Dutch are developing systems to sterilise the solution before it is recycled, and it seems likely that recirculating systems which are hybrids between standard rockwool and NFT systems will become more common.

## References

- Butters R 1989 *ADAS Hydroponics Survey*.  
Marks M J 1989 Use of sodium chloride for tomatoes grown in solution culture. *ADAS Hydroponics Survey*.  
Smith D L 1987 *Rockwool in Horticulture*. Grower Books, London.

## The Perlite Hydroponic System

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The perlite hydroponic growing system was initially devised and developed by the Soil Science Unit at the West of Scotland Agricultural College (Wilson *et al* 1984). More recently the system has been evaluated in ADAS trials at Stockbridge House Experimental Horticulture Station (EHS) and has found favour south of the border, particularly in Humberside and Lancashire. ADAS hydroponic survey figures show that there are 16 ha of tomatoes and 2 ha of cucumbers being grown on perlite in 1989, which represents 10% and 1% respectively of the hydroponic area of these crops in England and Wales (Butters 1989).

There are two systems in operation commercially. The 'gully' reservoir system is formed by sitting the perlite bag in individual reservoirs created in the bottom of a polythene gully, by a series of 3- to 4-cm-high polystyrene weirs (dams). The bags are slit vertically at the base to allow movement of solution between the outer reservoir and the bag. The outstanding feature of this system is the ease of watering management, which can be as infrequent as two or three times a day. This system has been widely adopted in Scotland and in Lancashire by growers new into hydroponic culture.

The 'bag' reservoir system is formed by slitting the bag horizontally 3–4 cm up from the base creating a reservoir within the bag; there is no outer gully or weir. The management requirements for this system are similar to those for rockwool with watering on a frequent basis (10–20 times a day) and for the driest bag. This system has the advantage that setting up and end-of-season turn-around are quicker than for the gully system. The bag reservoir system is the one most widely used by perlite growers in England, who have accurate watering systems and often previous experience with rockwool management.

The important feature of the system is the shallow depth of the nutrient solution in the base of the bag. Provided that this is maintained, water will be drawn up by capillary action to give a constant air/water balance in the perlite. As perlite is itself physically stable, perlite bags have been re-used for several seasons by some growers without sterilisation. However, this is not recommended due to disease risks.

ADAS work has shown that the most reliable method of monitoring nutrition is to take samples of nutrient solution from the reservoir maintained in the base of the

perlite bag. The reservoir solution has been shown to have the same analysis as that held on the perlite in the bottom half of the bag where the majority of roots are growing. A 'dipwell' system created by inserting porous plastic tubes into the bottom of 15–20 perlite bags has proved to be a quick and easy way for growers to take solution samples for analysis. The nutritional guidelines provided by ADAS for rockwool crops are also applicable to perlite.

Trials at Stockbridge House (1987 and 1988) compared the perlite and standard rockwool systems and found no significant differences in yield or quality (ADAS 1987 and 1988); though trends for higher early season yields from perlite were noted in both years.

Perlite can compete in terms of yields and economics with rockwool. Its major advantages are the freely draining nature of the medium and the ease of watering management, particularly for the gully system.

### References

- ADAS 1987 and 1988 Stockbridge House EHS. Internal ADAS Experiment Reports.  
Butters R E 1989 *ADAS Hydroponics Survey*.  
Wilson G C S, Hall D A, McGregor A J 1984 Perlite culture of tomatoes. *Technical Note 219*, West of Scotland Agricultural College.

## A Development Trial to Examine Conditions Likely to Lead to Blossom End Rot (BER) in Commercial, Hydroponically Fed Tomato Crops, 1985

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### Introduction

In the Eversham area in late April 1984 the developing fruit turned black on up to five trusses on many March-planted hydroponically grown tomato crops. The disorder coincided with a sudden change from prolonged dull to very bright weather. Affected plants were being grown in solution conductivities in the range 3–5 mS cm<sup>-1</sup>. Analysis of leaves and whole fruit showed calcium levels to be normal.

### Objective of the 1985 trial at Luddington EHS

The trial's objective was to induce BER in an April-planted hydroponically grown tomato crop under known environmental conditions; to establish analytically that BER caused by calcium deficiency can be related to calcium levels in the distal end of

the fruit; and to provide information towards a predictive technique for advice on the potential development of BER in commercial crops.

### *Method*

Plants of the variety Sonatine (Pinetree Vandenburg) propagated in 0.4-litre rockwool cubes were placed into 30-m-long nutrient film (NFT) channels on 17 April—5 days after the commencement of flowering. The nutrient solution in each of three unreplicated catchment tanks (each supplying three channels) was maintained throughout the summer at conductivities of 2, 4 and 8 mS cm<sup>-1</sup>. The formulation of these solutions was based on the recommendations now incorporated into ADAS Pamphlet 3174 (ADAS 1988) to give conductivities up to 3 mS cm<sup>-1</sup>. An additional solution of calcium nitrate (5 kg per 100 litres) and potassium nitrate (8 kg per 100 litres) was used to increase conductivities to 4 and 8 mS cm<sup>-1</sup>.

Half of the plants at each conductivity were grown under 42% shade continuously from 21 March until 15 May to see if a sudden change to bright light would induce the physiological stress suspected as being the cause of the 1984 BER problem. Half of the previously shaded and half of the unshaded plants were enclosed at night in tents of thin, clear polythene from mid-May to see if an increased RH would reduce BER.

Minimum air temperatures from planting were 13°C night, 20°C day, with ventilation commencing at 23°C. Solution temperature was maintained at 20°C. On 24 May the solution temperature was increased to 25°C and the night air temperature reduced to 10°C to increase plant vigour.

Regular analyses of the solutions and leaves were made during the season to monitor plant nutrition. Analyses of green fruit with and without visible symptoms of BER were carried out monthly; the petiole and distal thirds were analysed separately for calcium and the distal third for potassium content.

### *Results*

No nutritional deficiency or toxicity was observed visually or by analysis from any treatment. The use of shading or polythene tents did not affect the incidence of BER.

BER was first seen on 3 June following the first prolonged period of bright weather since the trial commenced. The expression of BER was the normal blackening of the distal end of the fruit rather than the overall blackening seen in 1984. BER symptoms were seen on 15% of the fruit formed on trusses 2–5 on the plants grown at 8 mS cm<sup>-1</sup> in spite of levels of around 800 mg litre<sup>-1</sup> in the nutrient solution. The incidence of BER was negligible on later trusses, and on all trusses produced by plants grown at the two lower conductivities.

Fruit analysis showed that the:

- (a) calcium content of whole fruit was lower the higher the solution conductivity;
- (b) ratio of calcium in the petiole to the distal third of the fruit increased from ~ 3:1 where BER was absent to 4:1 when BER was present;
- (c) ratio of potassium to calcium in the distal end of the fruit increased from under 100:1 where BER was absent to at least 140:1 where BER was present.

### Conclusions

- (a) Tomato fruit should be considered to be non-transpiring tissue. A high concentration of nutrients surrounding the roots, particularly at night, will restrict water uptake and hence calcium transport to the developing fruit.
- (b) To predict the development of BER the petiole and distal thirds of the fruit should be analysed separately. The higher the ratio of calcium between the petiole and distal ends, and the higher the potassium to calcium ratio in the distal end, the greater the likelihood of BER developing.

### Reference

- ADAS 1988 Pamphlet 3174: Tomatoes: nutrition for rockwool and NFT culture. MAFF, London.

## Mechanism of Calcium Transport in Tomato

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It is generally agreed that the cause of local calcium deficiency in tomato plants is the inadequate supply of calcium to the rapidly growing organs (Ehret and Ho 1986). Most of the investigations in recent years have been concerned with the environmental effects on the accumulation of calcium in the affected organs (Bradfield and Guttridge 1984; Adams and El-Gizawy 1986), but the mechanism of calcium transport is still not adequately understood.

Tomato plants were grown in recirculating nutrient solution (NFT) with a range of salinities (ie electrical conductivity 3 to 8 mS cm<sup>-1</sup>). High salinity was raised either by enriching the solution with macronutrients or by adding NaCl. Some plants were fed with nutrient solution of high salinity (8 mS cm<sup>-1</sup>) during the day and of low salinity (3 mS cm<sup>-1</sup>) at night (8/3 mS treatment). The accumulation of calcium in the distal and remaining fruit tissue was measured throughout fruit development. The accumulation of calcium in these fruits was compared with that of magnesium, a phloem-mobile cation (Adams and Ho 1989; Ho and Adams 1989). In addition, the short-term effect of salinity was investigated by the distribution of <sup>45</sup>Ca in plants at constant or fluctuating salinity (Ho 1989). The long-term effect of salinity on fruit development was also assessed by the transport of <sup>45</sup>Ca inside detached fruit which had developed under different salinities.

The degree of reduction in the accumulation of calcium by fruit was in proportion to the salinity, whether the salinity was raised by macronutrients or by NaCl. A reduction in the uptake of calcium caused by osmotic stress was the primary cause of low calcium accumulation in the fruit. At the same salinity, distal fruit accumulated less calcium than the proximal fruit of the same truss. Fruit of later trusses accumulated more calcium than the early trusses when grown at low salinity but not at high salinity. Both the length and the calcium exchange capacity of the

xylem tissue affect the supply of calcium to the fruit. In contrast, the accumulation of magnesium was not reduced by salinity and the magnesium content of the distal fruit was greater than that of the proximal fruit. The difference in the accumulation of calcium and magnesium further supports the notion that salinity reduces xylem transport of calcium by tomato fruit. Fruit grown at fluctuating salinity ( $8/3 \text{ mS cm}^{-1}$ ) accumulated less calcium than those at constant average salinity of  $5.5 \text{ mS cm}^{-1}$ . Fluctuating salinity reduced the calcium content of the distal tissue more than did the constant salinity.

The uptake of  $^{45}\text{Ca}$  by a tomato plant was higher during the day than at night. At night, due to low transpiration, less  $^{45}\text{Ca}$  was transported to mature leaves but more to the fruit, while a great proportion of  $^{45}\text{Ca}$  was detained in the stem. The import of  $^{45}\text{Ca}$  by the young leaves was reduced substantially by high humidity at night but not during the day. Although the uptake of  $^{45}\text{Ca}$  by the  $8/3 \text{ mS}$  plant at night was as high as that by the  $3 \text{ mS}$  plant, the daily uptake of  $^{45}\text{Ca}$  by the  $8/3 \text{ mS}$  plant was still less than that by the  $5.5 \text{ mS}$  plant. Under identical uptake conditions, the transport of  $^{45}\text{Ca}$  to the distal half of the fruit detached from the  $8/3 \text{ mS}$  plant was less than that from the  $5.5 \text{ mS}$  plant. The resistance of calcium transport in the fruit developed at  $8/3 \text{ mS}$  appears to be greater than that at  $5.5 \text{ mS}$ . High salinity reduces both the daily uptake of calcium by the plant (short-term effect) and the xylem transport inside the fruit (long-term effect).

### References

- Adams P, El-Gizawy A M 1986 Effect of salinity and watering level on the calcium content of tomato. *Acta Hort* **190** 253–259.
- Adams P, Ho L C 1989 Effects of diurnal changes in the salinity on the yield, quality and Ca status of tomato. *J Hort Sci* in press.
- Bradfield E G, Guttridge C G 1984 Effects of night-time humidity and nutrient solution concentration on the calcium of tomato fruit. *Sci Hort* **22** 207–217.
- Ehret D L, Ho L C 1986 Translocation of calcium in relation to tomato fruit growth. *Ann Bot* **58** 679–688.
- Ho L C 1989 Environmental effect on the diurnal accumulation of  $^{45}\text{Ca}$  by young fruit and leaves of tomato plants. *Ann Bot* **63** 281–288.
- Ho L C, Adams P 1989 Effects of diurnal changes in the salinity of the nutrient solution on the accumulation of calcium by tomato fruit. *Ann Bot* (in press).

### Effective Use of Carbon Dioxide in the Glasshouse Environment

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The benefits of carbon dioxide ( $\text{CO}_2$ ) enrichment for the improvement of productivity in glasshouse crops was first demonstrated in Britain in the 1920s. However, it was only with the advent of cheap sources of  $\text{CO}_2$  of sufficient purity in the 1960s that it became commercially viable.

CO<sub>2</sub> enrichment is now used in the production of the majority of heated glasshouse crops including tomatoes, lettuce, cucumber, peppers, Ayr chrysanthemums and some pot plants. Its benefits to yield and quality are well documented. Between 1965 and 1984 some 500 ha of heated glasshouses (about one-third of the total heated area of protected cropping in England and Wales) has been equipped with facilities for CO<sub>2</sub> enrichment.

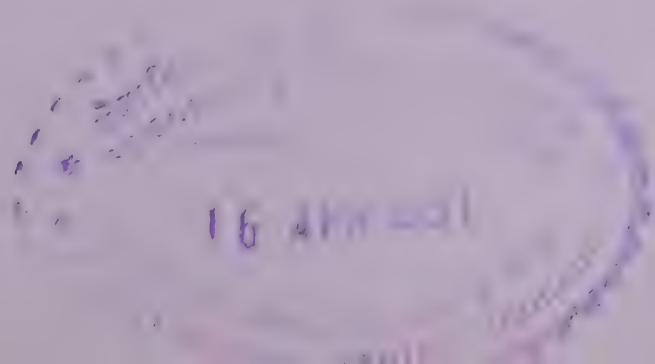
In most situations the glasshouse atmosphere is enriched to 1000 vpm following extensive experimentation with the major vegetable crops at IHR Littlehampton and the Lea Valley and Efford EHSs. Enrichment to ambient (335 vpm) has, however, been found economically justified during the summer period for both tomatoes and cucumbers.

There are two main commercial sources of CO<sub>2</sub>, pure gas stored in a pressure vessel, the direct combustion of hydrocarbon fuels within the glasshouse and the diversion of a proportion of the flue gas from a gas-fired heating boiler.

The selection of the most appropriate source of CO<sub>2</sub> for any particular nursery will depend on a number of factors. These include fuel availability, cropping cycle, capital and running costs, and size of production unit. For example, a large nursery growing cucumbers on rockwool might choose pure CO<sub>2</sub> in bulk for both units and summer enrichment where natural gas is not available to the site. On the other hand a smaller unit growing a lettuce and tomato rotation might choose to fire a hydrocarbon fuel direct.

Research at Lancaster University and IHR Littlehampton has demonstrated the potential for both visible crop damage and invisible losses of yield where contaminants such as oxides of nitrogen (NO<sub>x</sub>), sulphur dioxide and ethylene are released into the glasshouse atmosphere as a result of the combustion of hydrocarbon fuel. The use of low-sulphur kerosene and good maintenance of combustion equipment have largely eliminated crop damage from sulphur dioxide or products resulting from the incomplete combustion of hydrocarbons. However, heat produced in the burner flame makes it inevitable that some NO<sub>x</sub> will be released into the glasshouse atmosphere. As 0.5 vpm NO<sub>x</sub> is known to be present in flue gases containing 1000 vpm CO<sub>2</sub>, and Capron and Mansfield at Lancaster showed that exposure for a 20-h period to 0.5 vpm NO<sub>x</sub> depressed leaf net photosynthesis by approximately 30%, it is essential that enrichment should not exceed 1000 vpm CO<sub>2</sub>.

Carbon dioxide enrichment is likely to remain a key feature in attempts to maximise profits by controlling the glasshouse environment. The development of increasingly subtle environmental computer programs seeking to achieve the optimum combination of environmental factors for crop development, combined with more accurate sensing and control systems, intermittent instead of continuous injection, and improved distribution, are just some of the possibilities. Total environmental control must be the ultimate objective.









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